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GENZYME CORP. and THE REGENTS OF THE UNIVERSITY OF MICHIGAN

UNITED STATES DISTRICT COURT
DISTRICT OF NEW JERSEY

GENZYME CORP. and THE REGENTS OF THE UNIVERSITY OF MICHIGAN Plaintiffs, v. ZENARA PHARMA PRIVATE LIMITED, Defendant.	C.A. No.: ____________________ **(Filed Electronically)**

COMPLAINT FOR PATENT INFRINGEMENT

Plaintiffs Genzyme Corp. ("Genzyme") and The Regents of the University of Michigan ("U of M") (collectively, "Plaintiffs"), by way of their Complaint against Defendant Zenara Pharma Private Limited ("Zenara" or "Defendant"), allege as follows:

NATURE OF THE ACTION

1. This is a civil action for infringement of U.S. Patent No. 6,916,802 (the "'802 patent"), U.S. Patent No. 7,196,205 (the "'205 patent"), U.S. Patent No. 7,253,185 (the "'185 patent"), and U.S. Patent No. 7,615,573 (the "'573 patent") under the Patent Laws of the United States, 35 U.S.C. § 1 *et seq.*, including § 271, and for a declaratory judgment of infringement of the '802, '205, '185, and '573 patents under 28 U.S.C. §§ 2201 and 2202. This action arises out of Zenara's submission of Abbreviated New Drug Application ("ANDA") No. 212420 seeking approval to manufacture, use and/or sell a generic version of the pharmaceutical product CERDELGA® (eliglustat) capsules (84 mg) ("Zenara's ANDA product") prior to the expiration of the '802, '205, '185, and '573 patents. Plaintiffs seek injunctive relief against infringement, attorneys' fees, and any other relief the Court deems just and proper.

PARTIES

2. Plaintiff Genzyme is a corporation organized and existing under the laws of the Commonwealth of Massachusetts, having its principal place of business at 50 Binney Street, Cambridge, Massachusetts 02142. Genzyme is engaged in the business of research, development, manufacture, and sale of pharmaceutical products.

3. The Regents of the University of Michigan is a constitutional corporation of the State of Michigan, having a principal place of business at 1600 Huron Parkway, 2nd Floor, Ann Arbor, MI 48109.

4. Upon information and belief, Defendant Zenara is a corporation organized and existing under the laws of India, having its principal place of business at Plot 87-95, Phase III Industrial Development Area, Cherlapally, Hyderabad, 5000 51.

5. Upon information and belief, Zenara caused ANDA No. 212420 to be submitted to the United States Food and Drug Administration ("FDA") and seeks FDA approval of ANDA No. 212420. Upon information and belief, Biophore Pharma Inc., Zenara's U.S. agent, is a corporation organized and existing under the laws of New Jersey, having its principal place of business at 1, Deer Park, Suite F-8, Monmouth Junction, NJ 08852.

6. Upon information and belief, Zenara regularly transacts business throughout the United States and within New Jersey, including, but not limited to, marketing, distribution, sales, and/or offers to sell generic drugs.

JURISDICTION AND VENUE

7. This action arises under the Patent Laws of the United States and the Food and Drug Laws of the United States, Titles 35 and 21, United States Code. This Court has jurisdiction over the subject matter of this action under 28 U.S.C. §§ 1331 and 1338(a).

8. This Court has jurisdiction over Zenara because, *inter alia*, upon information and belief, Zenara has substantial, continuous, and systematic contacts within the State of New Jersey, and directly or indirectly manufactures, imports, markets, and sells generic drugs throughout the United States, including New Jersey, and New Jersey would be a destination of Zenara's ANDA product. Upon information and belief, Zenara filed ANDA No. 212420 through its U.S. agent Biophore Pharma Inc. which is organized and existing under the laws of the State of New Jersey and has its principal place of business in New Jersey. Upon information and belief, Zenara will market, distribute and/or sell Zenara's ANDA product in the United States, including

in New Jersey, upon approval of ANDA No. 212420, and will derive substantial revenue from the use or consumption of Zenara's ANDA product in the State of New Jersey.

9. In the alternative, this Court has jurisdiction over Zenara because the requirements of Federal Rule of Civil Procedure 4(k)(2) are met. This Court has jurisdiction over Zenara because, *inter alia*, this action arises under federal law, Zenara is a foreign defendant not subject to personal jurisdiction in the courts of any state, and Zenara has sufficient contacts with the United States as a whole, including but not limited to filing ANDAs with the FDA and manufacturing and selling generic pharmaceutical products that are distributed throughout the United States, such that this Court's exercise of jurisdiction over Zenara satisfies due process.

10. Venue is proper in this district for Zenara under 28 U.S.C. §§ 1391 and 1400(b) because, *inter alia*, Zenara is a corporation organized and existing under the laws of India, and may be sued in any judicial district. 28 U.S.C. § 1391(c).

CERDELGA®

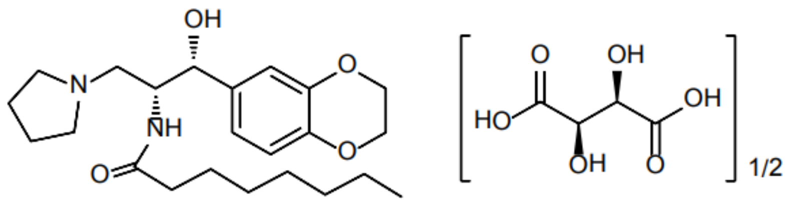
11. Genzyme is the holder of an approved New Drug Application ("NDA") No. 205494 for CERDELGA®, which the FDA approved on August 19, 2014.

12. CERDELGA® is a medication marketed and sold by Genzyme as 84 mg capsules for oral use. Genzyme received FDA approval to market CERDELGA® (eliglustat) for the long-term treatment of adult patients with Gaucher disease type 1 who are CYP2D6 extensive metabolizers (EMs), intermediate metabolizers (IMs), or poor metabolizers (PMs) as detected by an FDA-cleared test.

13. Eliglustat is the active ingredient in CERDELGA®. Eliglustat is a novel small molecule inhibitor of glucosylceramide synthase.

14. CERDELGA® capsules contain eliglustat as a hemitartrate salt (eliglustat tartrate), which can be referred to by the chemical name N-((1*R*,2*R*)-1-(2,3-

dihydrobenzo[b][1,4]dioxin-6-yl)-1-hydroxy-3-(pyrrolidin-1-yl)propan-2-yl)octanamide (2*R*,3*R*)-2,3-dihydroxysuccinate, and has the following chemical structure:



15. CERDELGA® was granted Orphan Drug Exclusivity for long-term treatment of adult patients with Gaucher disease type 1 who are CYP2D6 extensive metabolizers (EMs), intermediate metabolizers (IMs), or poor metabolizers (PMs) as detected by an FDA-cleared test.

THE PATENTS-IN-SUIT

16. On July 12, 2005, the ’802 patent, titled “Amino Ceramide-Like Compounds and Therapeutic Methods of Use,” was issued by the United States Patent and Trademark Office (“PTO”). A true and correct copy of the ’802 patent is attached as Exhibit A.

17. On March 27, 2007, the ’205 patent, titled “Synthesis of UDP-Glucose: *N*-Acylsphingosine Glucosyltransferase Inhibitors,” was issued by the PTO. A true and correct copy of the ’205 patent is attached as Exhibit B.

18. On August 7, 2007, the ’185 patent, titled “Amino Ceramide-Like Compounds and Therapeutic Methods of Use,” was issued by the PTO. A true and correct copy of the ’185 patent is attached as Exhibit C.

19. On November 10, 2009, the ’573 patent, titled “Synthesis of UDP-Glucose: N-Acylsphingosine Glucosyltransferase Inhibitors,” was issued by the PTO. A true and correct copy of the ’573 patent is attached as Exhibit D.

20. Eliglustat and methods of treating Gaucher disease with eliglustat are covered by one or more of the claims of the ’802, ’205, ’185, and ’573 patents, which are all listed in the *Approved Drug Products with Therapeutic Equivalence Evaluations* (“Orange Book”) for NDA No. 205494.

21. The ’802, ’205, ’185, and ’573 patents are owned by Genzyme and U of M.

ZENARA’S ANDA NO. 212420

22. Plaintiffs received a letter dated December 27, 2018 from Zenara notifying Plaintiffs that Zenara had submitted ANDA No. 212420 to the FDA under section 505(j) of the Federal Food, Drug, and Cosmetic Act (“FDCA”) seeking approval to commercially manufacture, use, sell, and/or import Zenara’s ANDA product prior to the expiration of the ’802, ’205, ’185, and ’573 patents.

23. Upon information and belief, Zenara’s ANDA No. 212420 seeks FDA approval of Zenara’s ANDA product for long-term treatment of adult patients with Gaucher disease type 1 who are CYP2D6 extensive metabolizers (EMs), intermediate metabolizers (IMs), or poor metabolizers (PMs) as detected by an FDA-cleared test.

24. According to applicable regulations, the purpose of Zenara’s December 27, 2018 letter was to notify Plaintiffs that ANDA No. 212420 included a certification under 21 U.S.C. § 355(j)(2)(A)(vii)(IV) (“Paragraph IV Certification”) alleging that the claims of the ’802, ’205, ’185, and ’573 patents are invalid, unenforceable, and/or would not be infringed by the commercial manufacture, use, sale, offer for sale, and/or importation of Zenara’s ANDA product.

25. Included in the December 27, 2018 letter was a “Detailed Statement of the Factual and Legal Basis” in support of Zenara’s Paragraph IV Certification, alleging that the claims of the ’802, ’205, ’185, and ’573 patents were invalid as obvious under 35 U.S.C. § 103.

26. Upon information and belief, Zenara was aware of the ’802, ’205, ’185, and ’573 patents when Zenara notified Plaintiffs of its Paragraph IV Certification regarding the ’802, ’205, ’185, and ’573 patents.

27. Plaintiffs commenced this action within 45 days of receipt of Zenara’s December 27, 2018 letter.

COUNT I
INFRINGEMENT OF THE ’802 PATENT

28. Plaintiffs incorporate and reallege paragraphs 1-27 above, as if set forth specifically here.

29. Upon information and belief, Zenara submitted ANDA No. 212420 to the FDA under the provisions of 21 U.S.C. § 355(j).

30. Upon information and belief, Zenara’s ANDA No. 212420 seeks FDA approval to engage in the commercial manufacture, use, sale, and/or importation of Zenara’s ANDA product (generic eliglustat in 84 mg capsules for oral use) before the expiration of the ’802 patent.

31. Plaintiffs received a letter from Zenara dated December 27, 2018, purporting to be a Notice of Certification for ANDA No. 212420 under Section 505(j)(2)(B) of the FDCA, 21 U.S.C. § 355(j)(2)(B), and 21 C.F.R. § 314.95.

32. Zenara’s December 27, 2018 letter states that the active ingredient in Zenara’s ANDA product for which it seeks approval is eliglustat. Upon information and belief, Zenara’s ANDA No. 212420 seeks FDA approval of Zenara’s ANDA Product for the long-term treatment of adult patients with Gaucher disease type 1 who are CYP2D6 extensive metabolizers (EMs), intermediate metabolizers (IMs), or poor metabolizers (PMs) as detected by an FDA-cleared test.

33. Upon information and belief, Zenara's ANDA Product, if approved and marketed, will be accompanied by a product label that will induce physicians to treat a patient having a glycosphingolipidosis disorder, including Gaucher disease, comprising the step of administering to the patient a therapeutically effective amount of a composition comprising eliglustat, and thereby induce infringement of the methods of at least claims 6, 7, 13, and 14 of the '802 patent under 35 U.S.C. § 271(b). Plaintiffs are unaware of any substantial non-infringing uses of Zenara's ANDA Product aside from treating a patient having a glycosphingolipidosis disorder, including Gaucher disease, comprising the step of administering to the patient a therapeutically effective amount of a composition comprising eliglustat, and therefore the marketing of Zenara's ANDA Product will contribute to infringement of at least claims 6, 7, 13, and 14 of the '802 patent under 35 U.S.C. § 271(c).

34. Upon information and belief, Zenara made and included in its ANDA No. 212420 a Paragraph IV Certification stating that, in Zenara's opinion, the '802 patent is invalid, unenforceable and/or not infringed.

35. Zenara's submission of ANDA No. 212420 to obtain approval to engage in the commercial manufacture, use, sale, and/or importation of Zenara's ANDA product prior to the expiration of the '802 patent constituted an act of infringement under 35 U.S.C. § 271(e)(2)(A).

36. Zenara's commercial manufacture, use, sale, and/or importation of Zenara's ANDA product would infringe, either literally or under the doctrine of equivalents, at least claims 1, 2, 8 and 9 of the '802 patent, and the sale of such a product will induce and/or contribute to the infringement of at least claims 6, 7, 13, and 14 of the '802. Accordingly, unless enjoined by this Court, upon FDA approval of ANDA No. 212420, Zenara will make, use, offer to sell, or sell

Zenara's ANDA product within the United States, or will import Zenara's ANDA product into the United States, and will thereby infringe at least claims 1, 2, 6-9, 13, and 14 of the '802 patent.

37. Zenara had actual knowledge of the '802 patent prior to submission of ANDA No. 212420, and was aware that the filing of ANDA No. 212420 with the request for FDA approval prior to the expiration of the '802 patent would constitute an act of infringement of the '802 patent. Zenara had no reasonable basis for asserting that the commercial manufacture, use, offer for sale, or sale of Zenara's ANDA product will not infringe at least claims 1, 2, 6-9, 13, and 14 of the '802 patent.

38. Zenara's "Detailed Statement of the Factual and Legal Basis" in its December 27, 2018 letter lacks any contention that Zenara's ANDA product will not infringe at least claims 1, 2, 6-9, 13, and 14 of the '802 patent.

39. On information and belief, Zenara's statement of the factual and legal bases for its opinions regarding invalidity of the '802 patent lacks an objective good faith basis.

40. This case is exceptional, and Plaintiffs are entitled to an award of attorneys' fees under 35 U.S.C. § 285.

41. Plaintiffs will be irreparably harmed if Zenara is not enjoined from infringing the '802 patent. Plaintiffs do not have an adequate remedy at law, and considering the balance of hardships between Plaintiffs and Zenara, injunctive relief is warranted. Further, the public interest favors entry of an injunction.

COUNT II
DECLARATORY JUDGMENT OF INFRINGEMENT OF THE '802 PATENT

42. Plaintiffs incorporate and reallege paragraphs 1-41 above, as if set forth specifically here.

43. Upon information and belief, if ANDA No. 212420 is approved, Zenara's ANDA product will be made, offered for sale, sold, or otherwise distributed in the United States, including in the State of New Jersey, by or through Zenara and its affiliates. Zenara will therefore infringe at least claims 1, 2, 6-9, 13, and 14 of the '802 patent under 35 U.S.C. § 271.

44. Zenara's "Detailed Statement of the Factual and Legal Basis" in its December 27, 2018 letter lacks any contention that Zenara's ANDA product will not infringe at least claims 1, 2, 6-9, 13, and 14 of the '802 patent.

45. Upon information and belief, Zenara's infringing activity, including the commercial manufacture, use, offer to sell, sale, or importation of Zenara's ANDA product complained of herein will begin immediately after the FDA approves ANDA No. 212420. Any such conduct before the '802 patent expires will infringe at least claims 1, 2, 6-9, 13, and 14 of the '802 patent under 35 U.S.C. § 271(a)-(c).

46. There is a real, substantial, and continuing justiciable controversy between Plaintiffs and Zenara concerning liability for the infringement of the '802 patent for which this Court may grant declaratory relief consistent with Article III of the United States Constitution.

47. Plaintiffs will be substantially and irreparably harmed by Zenara's infringing activities unless those activities are enjoined by this Court. Plaintiffs have no adequate remedy at law.

48. This case is exceptional, and Plaintiffs are entitled to an award of attorneys' fees under 35 U.S.C. § 285.

COUNT III
INFRINGEMENT OF THE '205 PATENT

49. Plaintiffs incorporate and reallege paragraphs 1-48 above, as if set forth specifically here.

50. Upon information and belief, Zenara submitted ANDA No. 212420 to the FDA under the provisions of 21 U.S.C. § 355(j).

51. Upon information and belief, Zenara's ANDA No. 212420 seeks FDA approval to engage in the commercial manufacture, use, sale, and/or importation of Zenara's ANDA product (generic eliglustat in 84 mg capsules for oral use) before the expiration of the '205 patent.

52. Plaintiffs received a letter from Zenara dated December 27, 2018, purporting to be a Notice of Certification for ANDA No. 212420 under Section 505(j)(2)(B) of the FDCA, 21 U.S.C. § 355(j)(2)(B), and 21 C.F.R. § 314.95.

53. Zenara's December 27, 2018 letter states that the active ingredient in Zenara's ANDA product for which it seeks approval is eliglustat.

54. Upon information and belief, Zenara made and included in its ANDA No. 212420 a Paragraph IV Certification stating that, in Zenara's opinion, the '205 patent is invalid, unenforceable and/or not infringed.

55. Zenara's submission of ANDA No. 212420 to obtain approval to engage in the commercial manufacture, use, sale, and/or importation of Zenara's ANDA product prior to the expiration of the '205 patent constituted an act of infringement under 35 U.S.C. § 271(e)(2)(A).

56. Zenara's commercial manufacture, use, sale, and/or importation of Zenara's ANDA product would infringe, either literally or under the doctrine of equivalents, at least claims 1 and 3-9 of the '205 patent. Accordingly, unless enjoined by this Court, upon FDA approval of ANDA No. 212420, Zenara will make, use, offer to sell, or sell Zenara's ANDA product within the United States, or will import Zenara's ANDA product into the United States, and will thereby infringe at least claims 1 and 3-9 of the '205 patent.

57. Zenara had actual knowledge of the ’205 patent prior to submission of ANDA No. 212420, and was aware that the filing of ANDA No. 212420 with the request for FDA approval prior to the expiration of the ’205 patent would constitute an act of infringement of the ’205 patent. Zenara had no reasonable basis for asserting that the commercial manufacture, use, offer for sale, or sale of Zenara’s ANDA product will not infringe at least claims 1 and 3-9 of the ’205 patent.

58. Zenara’s “Detailed Statement of the Factual and Legal Basis” in its December 27, 2018 letter lacks any contention that Zenara’s ANDA product will not infringe at least claims 1 and 3-9 of the ’205 patent.

59. On information and belief, Zenara’s statement of the factual and legal bases for its opinions regarding invalidity of the ’205 patent lacks an objective good faith basis.

60. This case is exceptional, and Plaintiffs are entitled to an award of attorneys’ fees under 35 U.S.C. § 285.

61. Plaintiffs will be irreparably harmed if Zenara is not enjoined from infringing the ’205 patent. Plaintiffs do not have an adequate remedy at law, and considering the balance of hardships between Plaintiffs and Zenara, injunctive relief is warranted. Further, the public interest favors entry of an injunction.

COUNT IV
DECLARATORY JUDGMENT OF INFRINGEMENT OF THE ’205 PATENT

62. Plaintiffs incorporate and reallege paragraphs 1-61 above, as if set forth specifically here.

63. Upon information and belief, if ANDA No. 212420 is approved, Zenara’s ANDA product will be made, offered for sale, sold, or otherwise distributed in the United States,

including in the State of New Jersey, by or through Zenara and its affiliates. Zenara will therefore infringe at least claims 1 and 3-9 of the ’205 patent under 35 U.S.C. § 271.

64. Zenara’s “Detailed Statement of the Factual and Legal Basis” in its December 27, 2018 letter lacks any contention that Zenara’s ANDA product will not infringe at least claims 1 and 3-9 of the ’205 patent.

65. Upon information and belief, Zenara’s infringing activity, including the commercial manufacture, use, offer to sell, sale, or importation of Zenara’s ANDA product complained of herein will begin immediately after the FDA approves ANDA No. 212420. Any such conduct before the ’205 patent expires will infringe at least claims 1 and 3-9 of the ’205 patent under 35 U.S.C. § 271.

66. There is a real, substantial, and continuing justiciable controversy between Plaintiffs and Zenara concerning liability for the infringement of the ’205 patent for which this Court may grant declaratory relief consistent with Article III of the United States Constitution.

67. Plaintiffs will be substantially and irreparably harmed by Zenara’s infringing activities unless those activities are enjoined by this Court. Plaintiffs have no adequate remedy at law.

68. This case is exceptional, and Plaintiffs are entitled to an award of attorneys’ fees under 35 U.S.C. § 285.

COUNT V
INFRINGEMENT OF THE ’185 PATENT

69. Plaintiffs incorporate and reallege paragraphs 1-68 above, as if set forth specifically here.

70. Upon information and belief, Zenara submitted ANDA No. 212420 to the FDA under the provisions of 21 U.S.C. § 355(j).

71. Upon information and belief, Zenara's ANDA No. 212420 seeks FDA approval to engage in the commercial manufacture, use, sale, and/or importation of Zenara's ANDA product (generic eliglustat in 84 mg capsules for oral use) before the expiration of the '185 patent.

72. Plaintiffs received a letter from Zenara dated December 27, 2018, purporting to be a Notice of Certification for ANDA No. 212420 under Section 505(j)(2)(B) of the FDCA, 21 U.S.C. § 355(j)(2)(B), and 21 C.F.R. § 314.95.

73. Zenara's December 27, 2018 letter states that the active ingredient in Zenara's ANDA product for which it seeks approval is eliglustat.

74. Upon information and belief, Zenara made and included in its ANDA No. 212420 a Paragraph IV Certification stating that, in Zenara's opinion, the '185 patent is invalid, unenforceable and/or not infringed.

75. Zenara's submission of ANDA No. 212420 to obtain approval to engage in the commercial manufacture, use, sale, and/or importation of Zenara's ANDA product prior to the expiration of the '185 patent constituted an act of infringement under 35 U.S.C. § 271(e)(2)(A).

76. Zenara's commercial manufacture, use, sale, and/or importation of Zenara's ANDA product would infringe, either literally or under the doctrine of equivalents, claims 1-4 of the '185 patent. Accordingly, unless enjoined by this Court, upon FDA approval of ANDA No. 212420, Zenara will make, use, offer to sell, or sell Zenara's ANDA product within the United States, or will import Zenara's ANDA product into the United States, and will thereby infringe claims 1-4 of the '185 patent.

77. Zenara had actual knowledge of the '185 patent prior to submission of ANDA No. 212420, and was aware that the filing of ANDA No. 212420 with the request for FDA

approval prior to the expiration of the ’185 patent would constitute an act of infringement of the ’185 patent. Zenara had no reasonable basis for asserting that the commercial manufacture, use, offer for sale, or sale of Zenara’s ANDA product will not infringe claims 1-4 of the ’185 patent.

78. Zenara’s “Detailed Statement of the Factual and Legal Basis” in its December 27, 2018 letter lacks any contention that Zenara’s ANDA product will not infringe claims 1-4 of the ’185 patent.

79. On information and belief, Zenara’s statement of the factual and legal bases for its opinions regarding invalidity of the ’185 patent lacks an objective good faith basis.

80. This case is exceptional, and Plaintiffs are entitled to an award of attorneys’ fees under 35 U.S.C. § 285.

81. Plaintiffs will be irreparably harmed if Zenara is not enjoined from infringing the ’185 patent. Plaintiffs do not have an adequate remedy at law, and considering the balance of hardships between Plaintiffs and Zenara, injunctive relief is warranted. Further, the public interest favors entry of an injunction.

COUNT VI
DECLARATORY JUDGMENT OF INFRINGEMENT OF THE ’185 PATENT

82. Plaintiffs incorporate and reallege paragraphs 1-81 above, as if set forth specifically here.

83. Upon information and belief, if ANDA No. 212420 is approved, Zenara’s ANDA product will be made, offered for sale, sold, or otherwise distributed in the United States, including in the State of New Jersey, by or through Zenara and its affiliates. Zenara will therefore infringe claims 1-4 of the ’185 patent under 35 U.S.C. § 271.

84. Zenara's "Detailed Statement of the Factual and Legal Basis" in its December 27, 2018 letter lacks any contention that Zenara's ANDA product will not infringe claims 1-4 of the '185 patent.

85. Upon information and belief, Zenara's infringing activity, including the commercial manufacture, use, offer to sell, sale, or importation of Zenara's ANDA product complained of herein will begin immediately after the FDA approves ANDA No. 212420. Any such conduct before the '185 patent expires will infringe claims 1-4 of the '185 patent under 35 U.S.C. § 271.

86. There is a real, substantial, and continuing justiciable controversy between Plaintiffs and Zenara concerning liability for the infringement of the '185 patent for which this Court may grant declaratory relief consistent with Article III of the United States Constitution.

87. Plaintiffs will be substantially and irreparably harmed by Zenara's infringing activities unless those activities are enjoined by this Court. Plaintiffs have no adequate remedy at law.

88. This case is exceptional, and Plaintiffs are entitled to an award of attorneys' fees under 35 U.S.C. § 285.

COUNT VII
INFRINGEMENT OF THE '573 PATENT

89. Plaintiffs incorporate and reallege paragraphs 1-88 above, as if set forth specifically here.

90. Upon information and belief, Zenara submitted ANDA No. 212420 to the FDA under the provisions of 21 U.S.C. § 355(j).

91. Upon information and belief, Zenara's ANDA No. 212420 seeks FDA approval to engage in the commercial manufacture, use, sale, and/or importation of Zenara's

ANDA product (generic eliglustat in 84 mg capsules for oral use) before the expiration of the ’573 patent.

92. Plaintiffs received a letter from Zenara dated December 27, 2018, purporting to be a Notice of Certification for ANDA No. 212420 under Section 505(j)(2)(B) of the FDCA, 21 U.S.C. § 355(j)(2)(B), and 21 C.F.R. § 314.95.

93. Zenara’s December 27, 2018 letter states that the active ingredient in Zenara’s ANDA product for which it seeks approval is eliglustat. Upon information and belief, Zenara’s ANDA No. 212420 seeks FDA approval of Zenara’s ANDA Product for the long-term treatment of adult patients with Gaucher disease type 1 who are CYP2D6 extensive metabolizers (EMs), intermediate metabolizers (IMs), or poor metabolizers (PMs) as detected by an FDA-cleared test.

94. Upon information and belief, Zenara’s ANDA Product, if approved and marketed, will be accompanied by a product label that will induce physicians to inhibit glucosylceramide synthase or lower glycosphingolipid concentrations in a subject in need thereof, or treat Gaucher disease, comprising administering to the subject an effective amount of eliglustat, and thereby induce infringement of the methods of at least claims 1-5 and 21-25 of the ’573 patent under 35 U.S.C. § 271(b). Plaintiffs are unaware of any substantial non-infringing uses of Zenara’s ANDA Product aside from inhibiting glucosylceramide synthase or lowering glycosphingolipid concentrations in a subject in need thereof, or treating Gaucher disease, comprising administering to the subject an effective amount of eliglustat, and therefore the marketing of Zenara’s ANDA Product will contribute to infringement of at least claims 1-5 and 21-25 of the ’573 patent under 35 U.S.C. § 271(c).

95. Upon information and belief, Zenara made and included in its ANDA No. 212420 a Paragraph IV Certification stating that, in Zenara's opinion, the '573 patent is invalid, unenforceable and/or not infringed.

96. Zenara's submission of ANDA No. 212420 to obtain approval to engage in the commercial manufacture, use, sale, and/or importation of Zenara's ANDA product prior to the expiration of the '573 patent constituted an act of infringement under 35 U.S.C. § 271(e)(2)(A).

97. Zenara's commercial offer for sale or sale of Zenara's ANDA product would induce and/or contribute to infringement of at least claims 1-5 and 21-25 of the '573 patent. Accordingly, unless enjoined by this Court, upon FDA approval of ANDA No. 212420, Zenara will offer to sell or sell Zenara's ANDA product within the United States and will thereby infringe at least claims 1-5 and 21-25 of the '573 patent.

98. Zenara had actual knowledge of the '573 patent prior to submission of ANDA No. 212420, and was aware that the filing of ANDA No. 212420 with the request for FDA approval prior to the expiration of the '573 patent would constitute an act of infringement of the '573 patent. Zenara had no reasonable basis for asserting that the commercial manufacture, use, offer for sale, or sale of Zenara's ANDA product will not infringe at least claims 1-5 and 21-25 of the '573 patent.

99. Zenara's "Detailed Statement of the Factual and Legal Basis" in its December 27, 2018 letter lacks any contention that Zenara's ANDA product will not infringe at least claims 1-5 and 21-25 of the '573 patent.

100. On information and belief, Zenara's statement of the factual and legal bases for its opinions regarding invalidity of the '573 patent lacks an objective good faith basis.

101. This case is exceptional, and Plaintiffs are entitled to an award of attorneys' fees under 35 U.S.C. § 285.

102. Plaintiffs will be irreparably harmed if Zenara is not enjoined from infringing the '573 patent. Plaintiffs do not have an adequate remedy at law, and considering the balance of hardships between Plaintiffs and Zenara, injunctive relief is warranted. Further, the public interest favors entry of an injunction.

COUNT VIII
DECLARATORY JUDGMENT OF INFRINGEMENT OF THE '573 PATENT

103. Plaintiffs incorporate and reallege paragraphs 1-102 above, as if set forth specifically here.

104. Upon information and belief, if ANDA No. 212420 is approved, Zenara's ANDA product will be offered for sale, sold, or otherwise distributed in the United States, including in the State of New Jersey, by or through Zenara and its affiliates. Zenara will therefore infringe at least claims 1-5 and 21-25 of the '573 patent under 35 U.S.C. § 271(b)-(c).

105. Zenara's "Detailed Statement of the Factual and Legal Basis" in its December 27, 2018 letter lacks any contention that Zenara's ANDA product will not infringe at least claims 1-5 and 21-25 of the '573 patent.

106. Upon information and belief, Zenara's infringing activity, including the commercial manufacture, use, offer to sell, sale, or importation of Zenara's ANDA product complained of herein will begin immediately after the FDA approves ANDA No. 212420. Any such conduct before the '573 patent expires will infringe at least claims 1-5 and 21-25 of the '573 patent under 35 U.S.C. § 271.

107. There is a real, substantial, and continuing justiciable controversy between Plaintiffs and Zenara concerning liability for the infringement of the ’573 patent for which this Court may grant declaratory relief consistent with Article III of the United States Constitution.

108. Plaintiffs will be substantially and irreparably harmed by Zenara’s infringing activities unless those activities are enjoined by this Court. Plaintiffs have no adequate remedy at law.

109. This case is exceptional, and Plaintiffs are entitled to an award of attorneys’ fees under 35 U.S.C. § 285.

REQUEST FOR RELIEF

WHEREFORE, Plaintiffs respectfully request the following relief:

A. A judgment that the claims of the ’802, ’205, ’185, and ’573 patents were infringed by Zenara’s submission of ANDA No. 212420 under 35 U.S.C. § 271(e)(2)(A), and that the commercial manufacture, use, offer for sale, sale, and/or importation of Zenara’s ANDA product prior to the expiration of the ’802, ’205, ’185, and ’573 patents will constitute an act of infringement of at least 1, 2, 6-9, 13, and 14 of the ’802 patent, claims 1 and 3-9 of the ’205 patent, claims 1-4 of the ’185 patent, and claims 1-5 and 21-25 of the ’573 patent;

B. A declaratory judgment that under 35 U.S.C. § 271, Zenara’s commercial manufacture, use, offer for sale, or sale in, or importation into, the United States of Zenara’s ANDA product would constitute infringement of at least 1, 2, 6-9, 13, and 14 of the ’802 patent, claims 1 and 3-9 of the ’205 patent, claims 1-4 of the ’185 patent, and claims 1-5 and 21-25 of the ’573 patent;

C. An order permanently enjoining Zenara, its affiliates, subsidiaries, and each of its officers, agents, servants and employees and those acting in privity or concert with it, from making, using, offering to sell, or selling in the United States, or importing into the United States, Zenara’s

ANDA product until after the expiration of the ’802, ’205, ’185, and ’573 patents, including any extensions and/or additional periods of exclusivity to which Plaintiffs are or become entitled;

D. An order under 35 U.S.C. § 271 (e)(4)(A) that the effective date of any FDA approval of ANDA No. 212420 shall be a date that is not earlier than the expiration date of the ’802, ’205, ’185, and ’573 patents, inclusive of any extensions and/or additional periods of exclusivity to which Plaintiffs are or become entitled;

E. A declaration under 28 U.S.C. § 2201 that if Zenara, its officers, agents, servants, employees, licensees, representatives, and attorneys, and any other persons acting or attempting to act in active concert or participation with it or acting on its behalf, engages in the commercial manufacture, use, offer for sale, sale, and/or importation of the product described in ANDA No. 212420, it will constitute an act of infringement of the ’802, ’205, ’185, and ’573 patents;

F. A judgment that the claims of the ’802, ’205, ’185, and ’573 patents are valid and enforceable;

G. An award of damages or other relief, pursuant to 35 U.S.C. § 271(e)(4)(C), if Zenara engages in the commercial manufacture, use, offer for sale, sale, and/or importation of Zenara’s ANDA product, or any product that infringes the ’802, ’205, ’185, and ’573 patents, prior to the expiration of the ’802, ’205, ’185, and ’573 patents, inclusive of any extensions and/or additional periods of exclusivity to which Plaintiffs are or become entitled;

H. A declaration that this is an exceptional case under 35 U.S.C. §§ 285 and 271(e)(4) and awarding Plaintiffs’ costs, expenses, and disbursements in this action, including reasonable attorney fees; and

I. An award of such other and further relief as this Court deems just and proper.

Dated: February 8, 2019

WALSH PIZZI O'REILLY FALANGA LLP

s/ Liza M. Walsh

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RULE 11.2 CERTIFICATION

We hereby certify that, to the best of our knowledge, the matter in controversy is related to the following actions:

- *Genzyme Corporation and The Regents of the University of Michigan v. Apotex Corp. and Apotex Inc.*, 1-18-cv-01795 (D. Del.);
- *Genzyme Corporation and The Regents of the University of Michigan v. Teva Pharmaceuticals USA, LLC*, 1-18-cv-01796 (D. Del.);
- *Genzyme Corporation and The Regents of the University of Michigan v. Dr. Reddy's Laboratories, Inc. and Dr. Reddy's Laboratories, Ltd.*, 1-18-cv-01839 (D. Del.);
- *Genzyme Corporation and The Regents of the University of Michigan v. Cipla Ltd.*, 1-18-cv-01838 (D. Del.);
- *Genzyme Corporation and The Regents of the University of Michigan v. Aizant Drug Research Solutions Private Ltd.*, 1-18-cv-1837 (D. Del.); and
- *Genzyme Corporation and The Regents of the University of Michigan v. Zenara Pharma Private Limited*, 1:19-cv-00264 (D. Del.).

Dated: February 8, 2019

WALSH PIZZI O'REILLY FALANGA LLP

s/ Liza M. Walsh

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RULE 201.1 CERTIFICATION

We hereby certify that the above-captioned matter is not subject to compulsory arbitration in that the Plaintiffs seek, *inter alia*, injunctive relief.

Dated: February 8, 2019

WALSH PIZZI O'REILLY FALANGA LLP

s/ Liza M. Walsh

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EXHIBIT A

US006916802B2

(12) **United States Patent**
Shayman et al.

(10) **Patent No.: US 6,916,802 B2**
(45) **Date of Patent: Jul. 12, 2005**

(54) **AMINO CERAMIDE-LIKE COMPOUNDS AND THERAPEUTIC METHODS OF USE**

(75) Inventors: **James A. Shayman**, Ann Arbor, MI (US); **David J. Harris**, Lexington, MA (US); **Craig Siegel**, Woburn, MA (US); **Carol A. Nelson**, Westford, MA (US); **Diane P. Copeland**, North Billerica, MA (US)

(73) Assignees: **Genzyme Corporation**, Cambridge, MA (US); **The Regents of the University of Michigan**, Ann Arbor, MI (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **10/839,497**

(22) Filed: **May 5, 2004**

(65) **Prior Publication Data**

US 2005/0049235 A1 Mar. 3, 2005

Related U.S. Application Data

(63) Continuation of application No. 10/134,315, filed on Apr. 29, 2002, now abandoned.

(51) **Int. Cl.**[7] **A61K 31/40**; A61K 31/445; A61K 31/5375; C07D 207/16; C07D 211/34

(52) **U.S. Cl.** **514/210.01**; 514/233.8; 514/237.8; 514/315; 514/321; 514/331; 514/422; 514/428; 544/148; 544/168; 546/197; 546/233; 548/528; 548/568; 548/950

(58) **Field of Search** 544/148, 168; 546/197, 233; 548/528, 568, 590; 514/210.01, 233.8, 237.8, 315, 321, 331, 422, 428

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Primary Examiner—Richard L. Raymond
(74) *Attorney, Agent, or Firm*—Hamilton, Brook, Smith & Reynolds, P.C.

(57) **ABSTRACT**

The present invention provides amino ceramide-like compounds which inhibit glucosyl ceramide (GlyCer) formation by inhibiting the enzyme GlyCer synthase, thereby lowering the level of glycosphingolipids. The compounds of the present invention have improved GlcCer synthase inhibition activity and are therefore useful in therapeutic methods for treating various conditions and diseases associated with altered glycosphingolipid levels.

14 Claims, 12 Drawing Sheets

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Figure 1

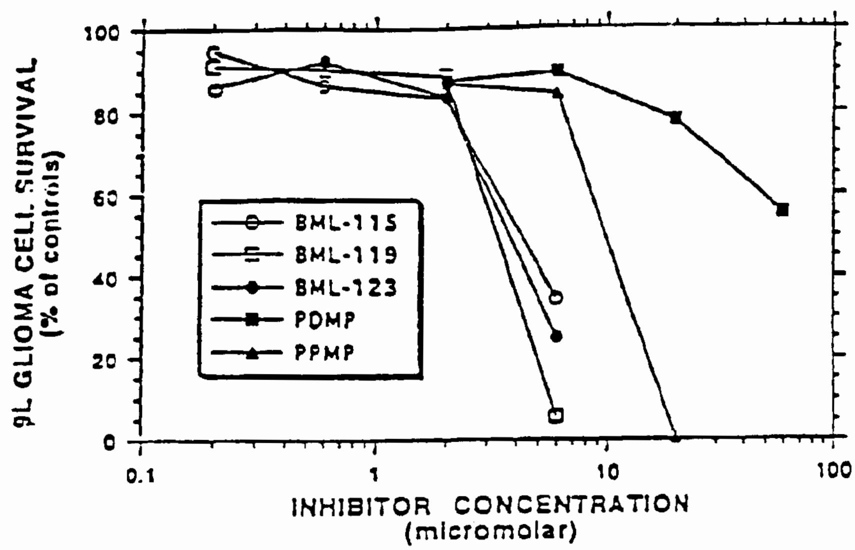


Figure 2

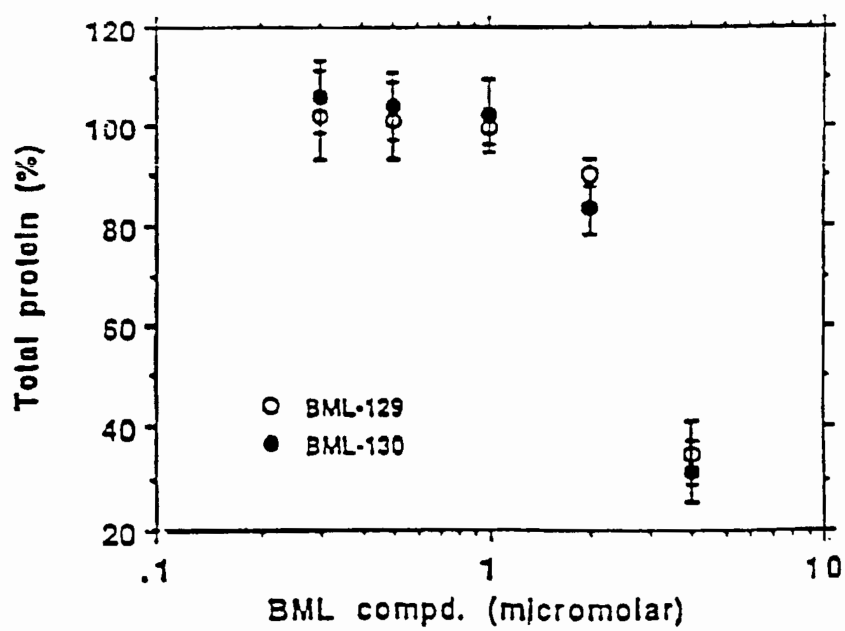
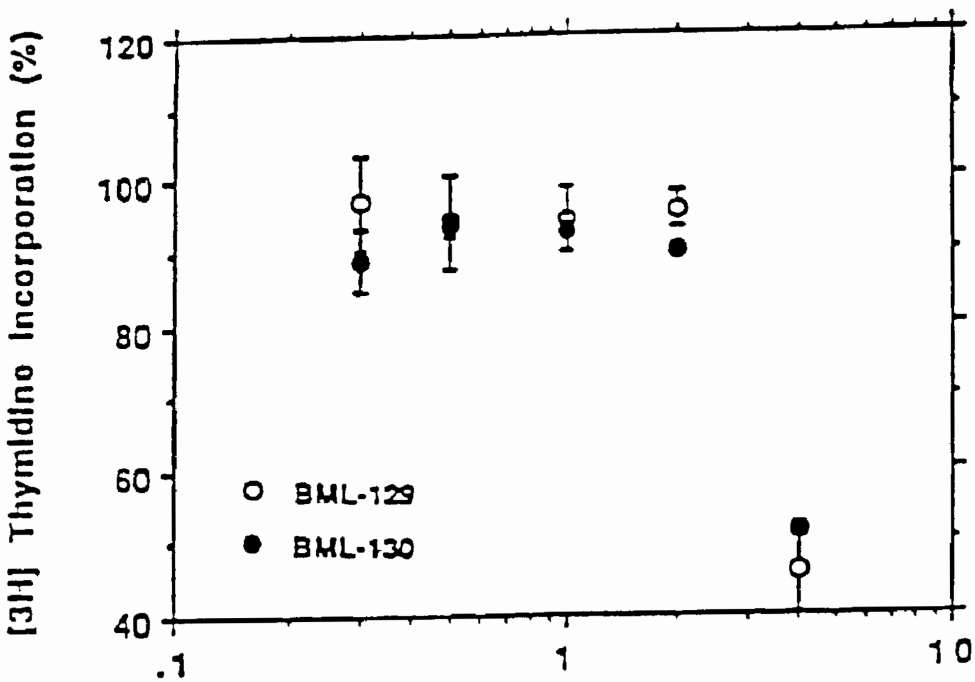


Figure 3


[3H] Thymidine Incorporation (%)
120
100
80
60
40
.1
1
10
BML-129
BML-130

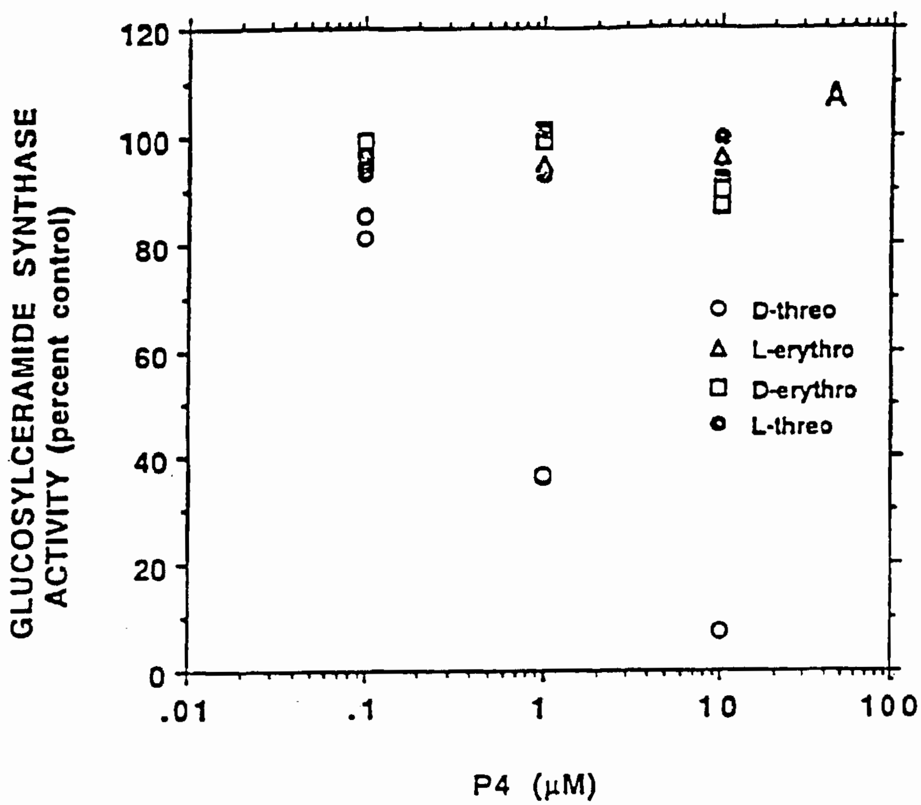
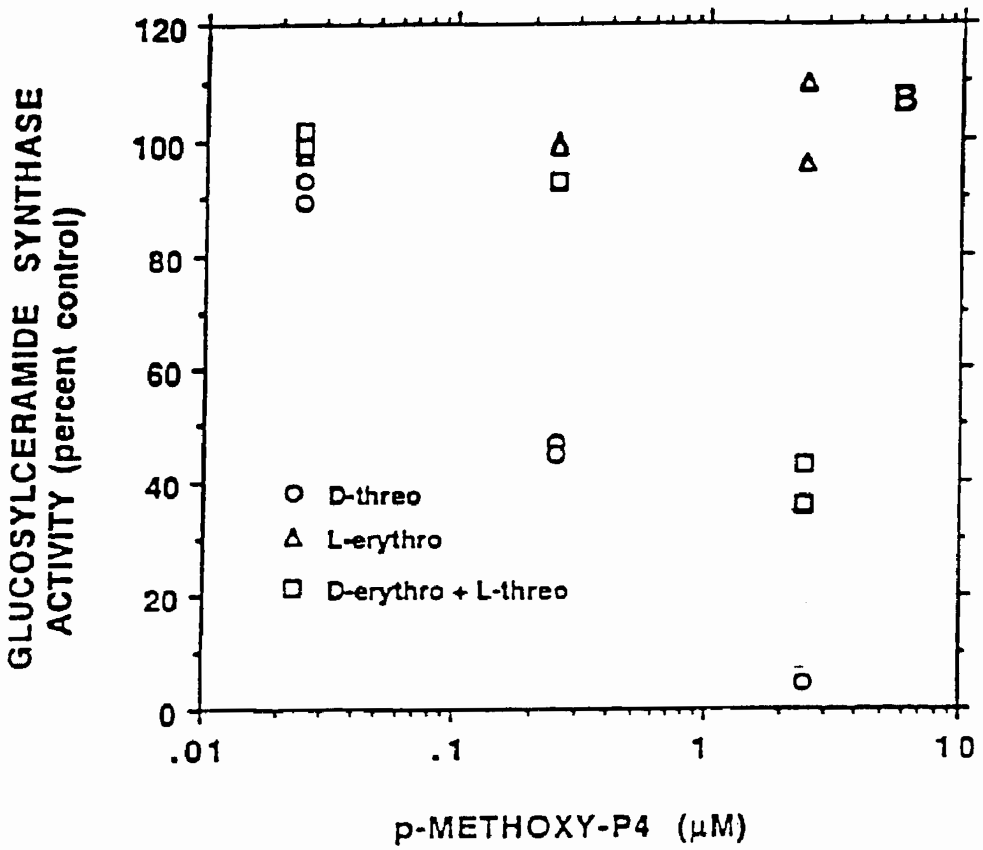

120
100
80
60
40
20
0
.01
.1
1
10
100
A
D-threo
L-erythro
D-erythro
L-threo
GLUCOSYLCERAMIDE SYNTHASE ACTIVITY (percent control)
P4 (μM)

Figure 4A



B
GLUCOSYLCERAMIDE SYNTHASE ACTIVITY (percent control)
120
100
80
60
40
20
0
.01
.1
1
10
p-METHOXY-P4 (μM)
D-threo
L-erythro
D-erythro + L-threo

Figure 4B

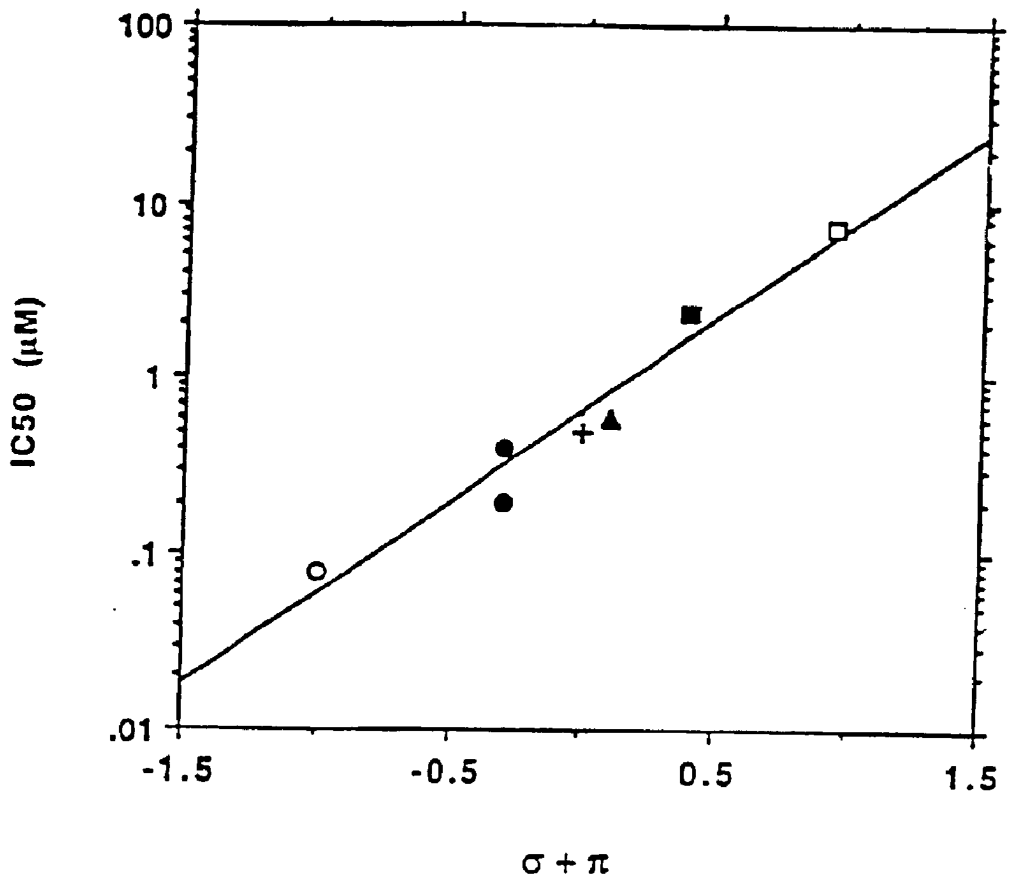


Figure 5

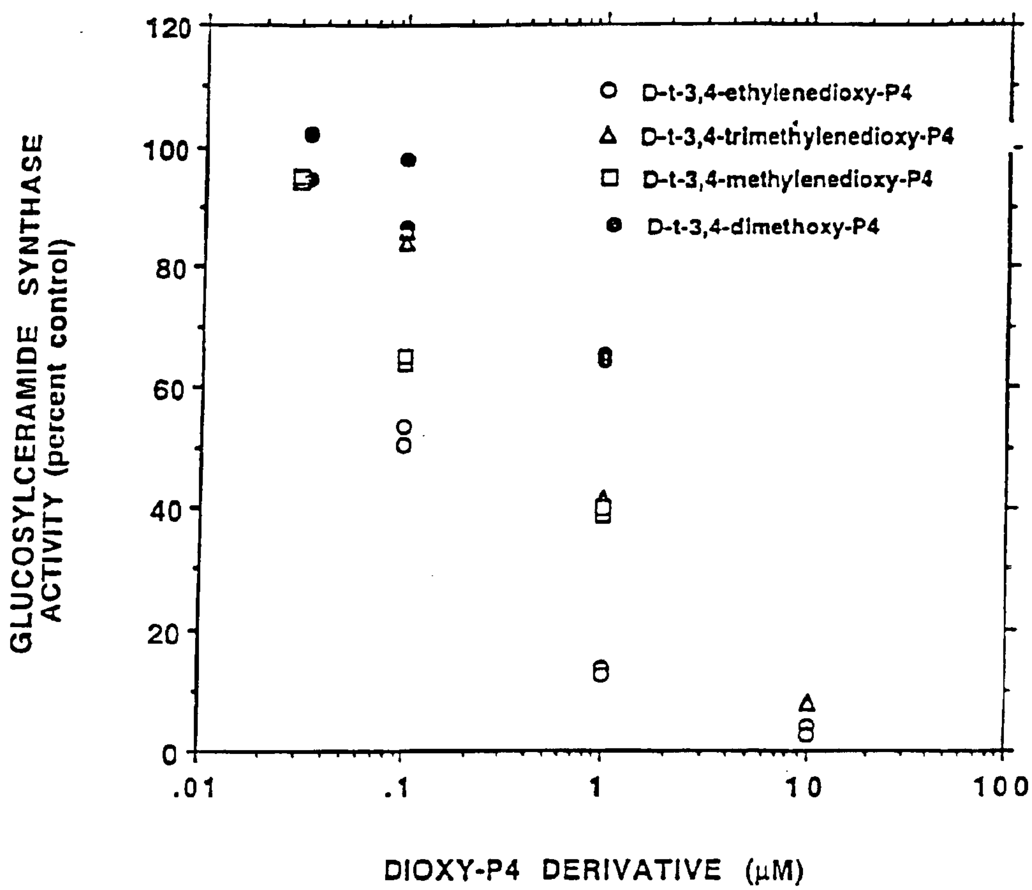
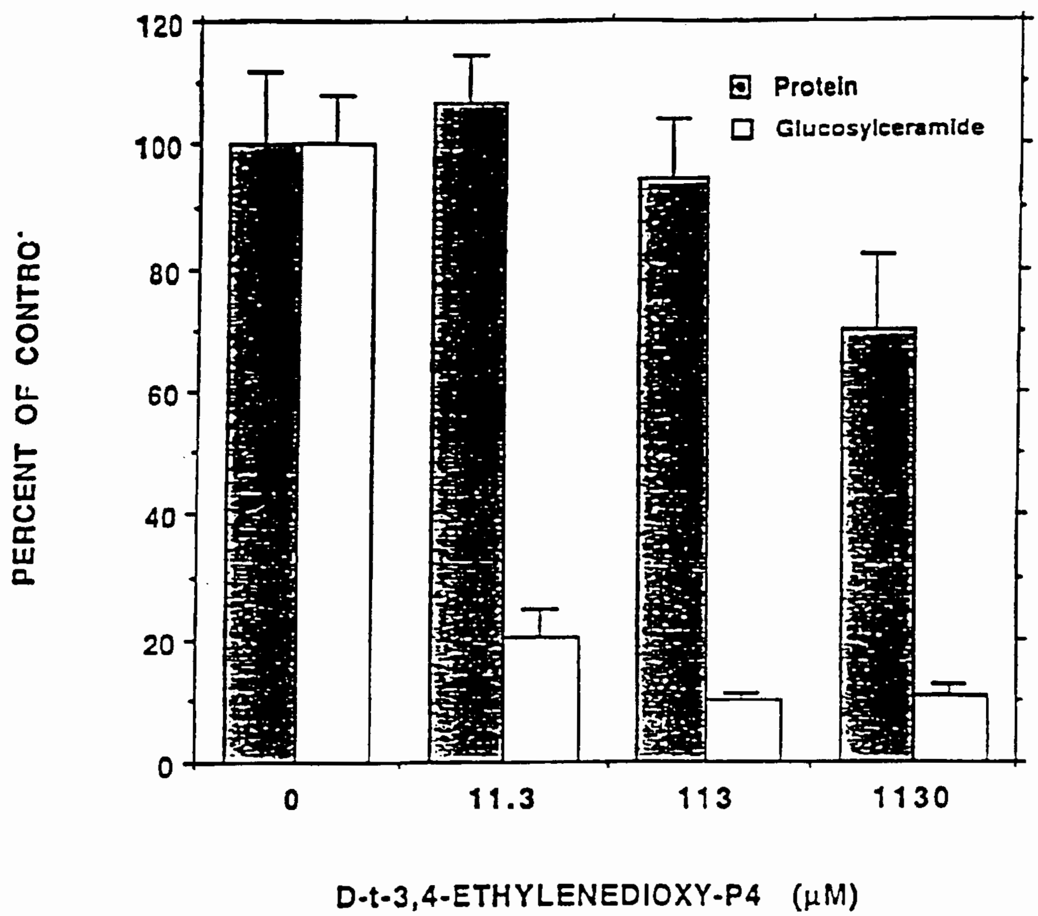

120
100
80
60
40
20
0
.01
.1
1
10
100
GLUCOSYLCERAMIDE SYNTHASE ACTIVITY (percent control)
DIOXY-P4 DERIVATIVE (μM)
D-t-3,4-ethylenedioxy-P4
D-t-3,4-trimethylenedioxy-P4
D-t-3,4-methylenedioxy-P4
D-t-3,4-dimethoxy-P4

Figure 6



120
100
80
60
40
20
0
PERCENT OF CONTRO'
Protein
Glucosylceramide
0
11.3
113
1130
D-t-3,4-ETHYLENEDIOXY-P4 (µM)

Figure 7

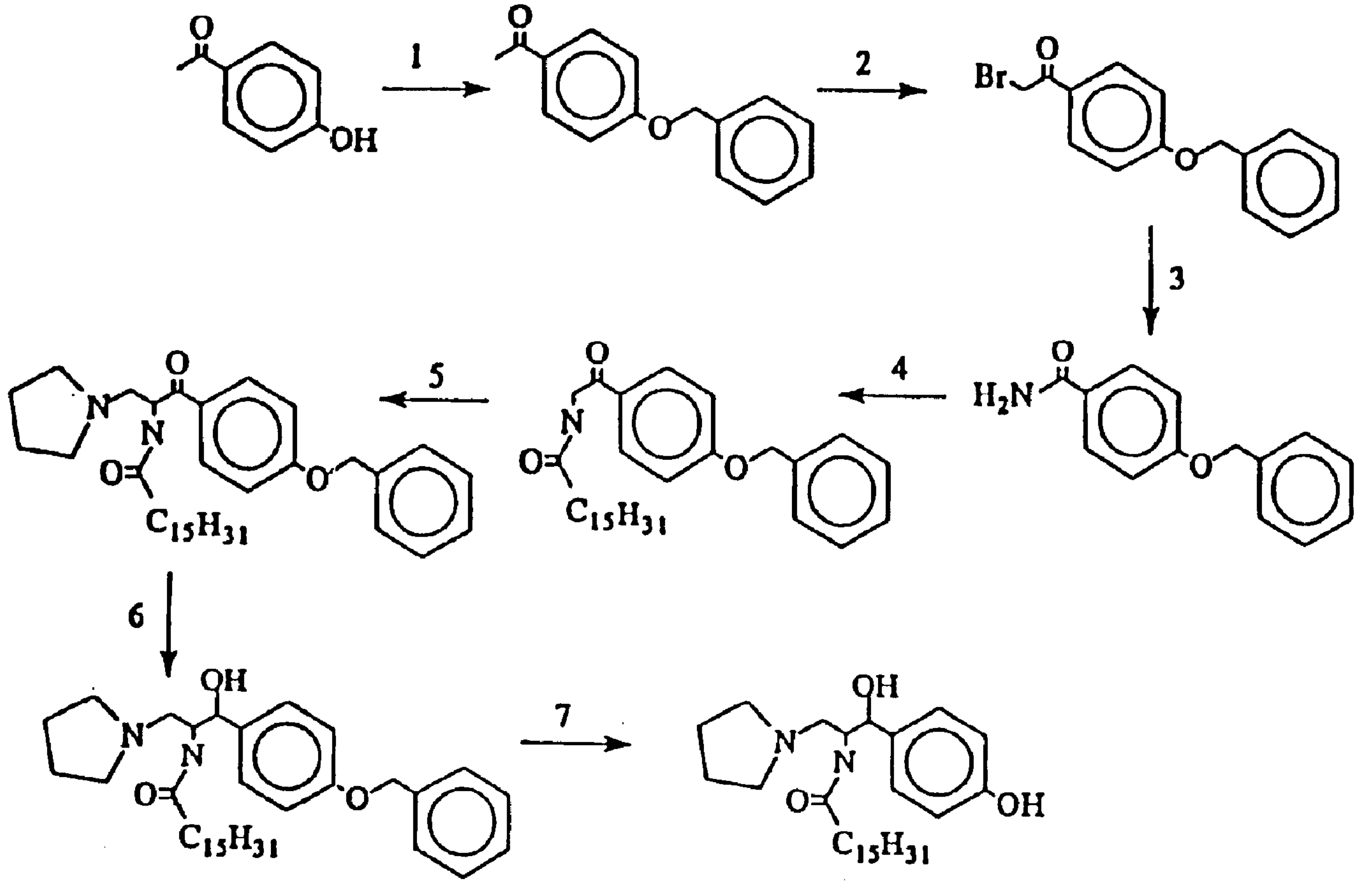
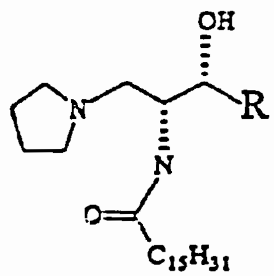
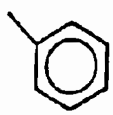


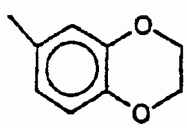
Figure 8



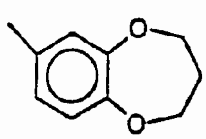
R=



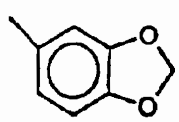
D-*threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4)



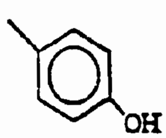
D-*threo*-1-(3',4'-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol



D-*threo*-1-(3',4'-trimethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol



D-*threo*-1-(3',4'-methylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol



D-*threo*-4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4)

Figure 9

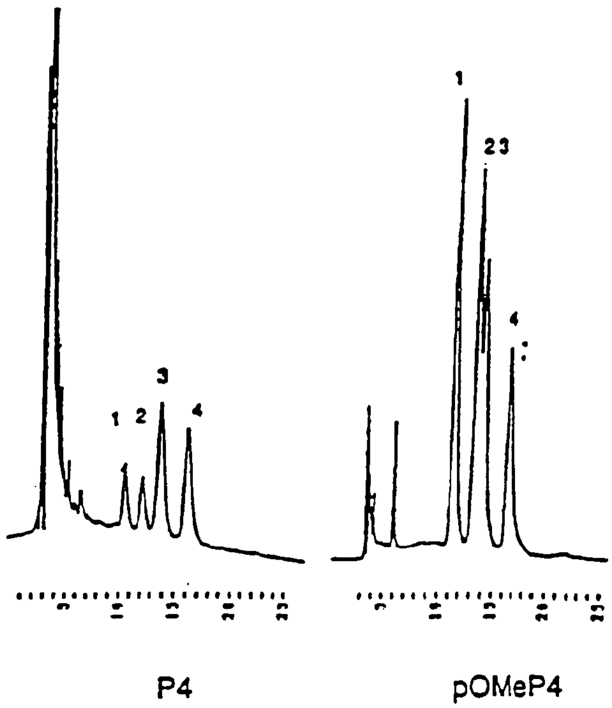


Figure 10

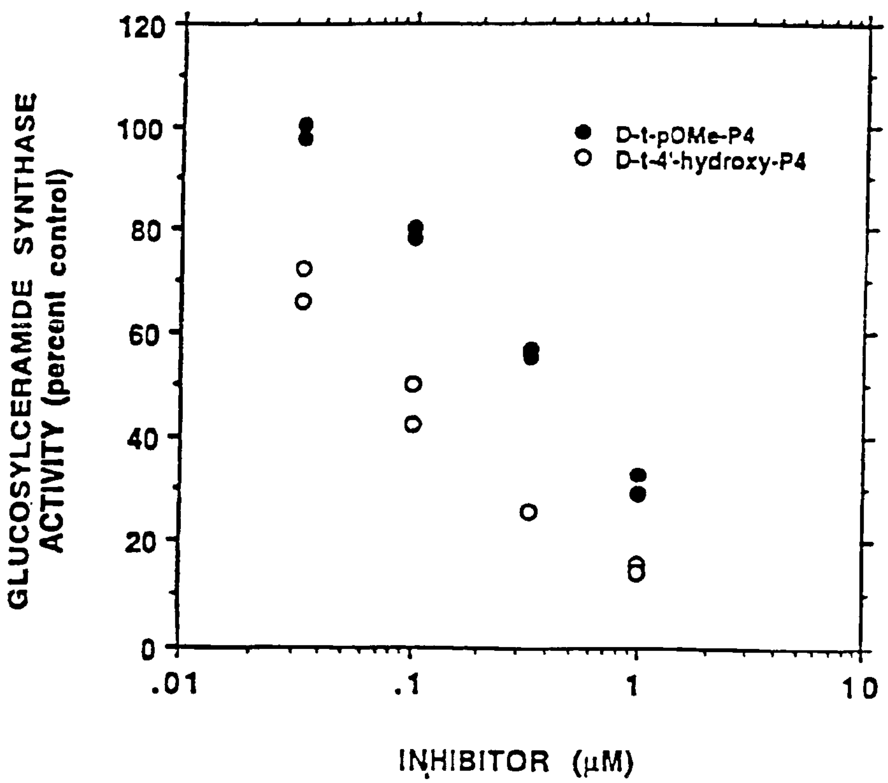


Figure 11

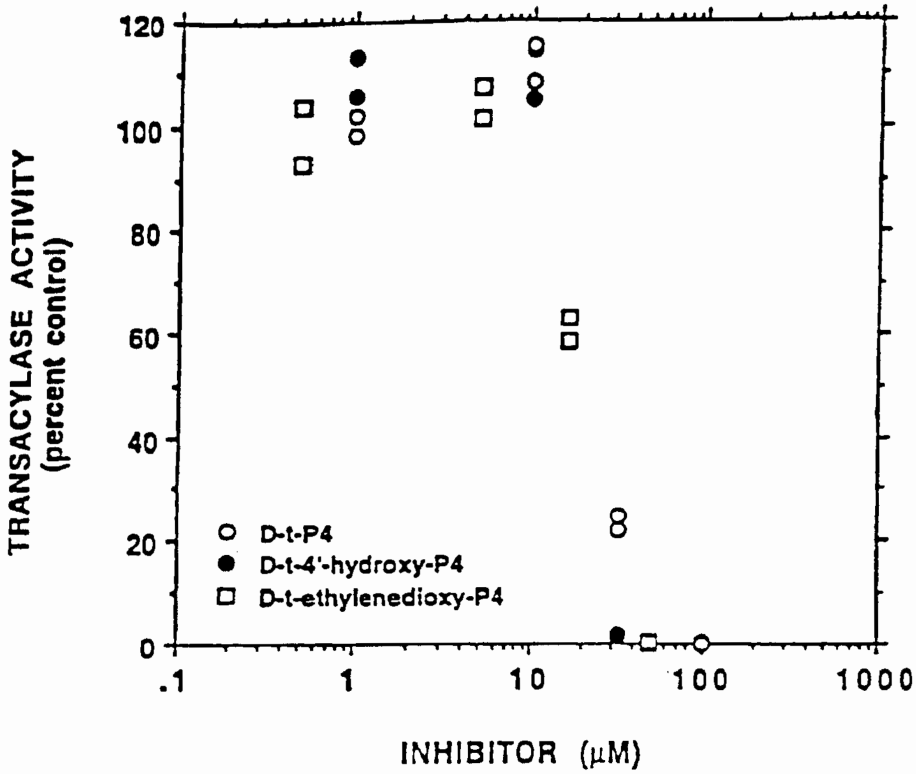


Figure 12

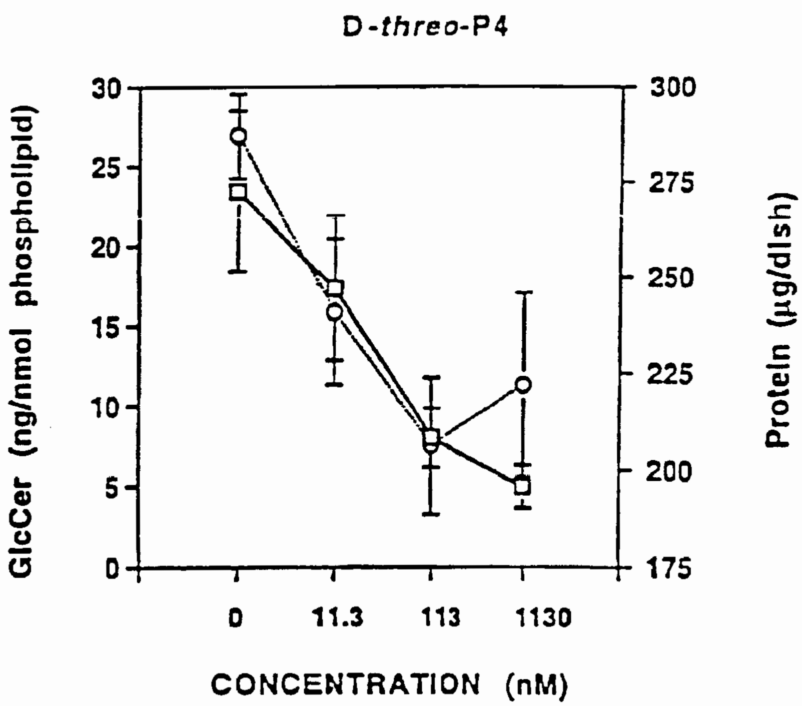


Figure 13

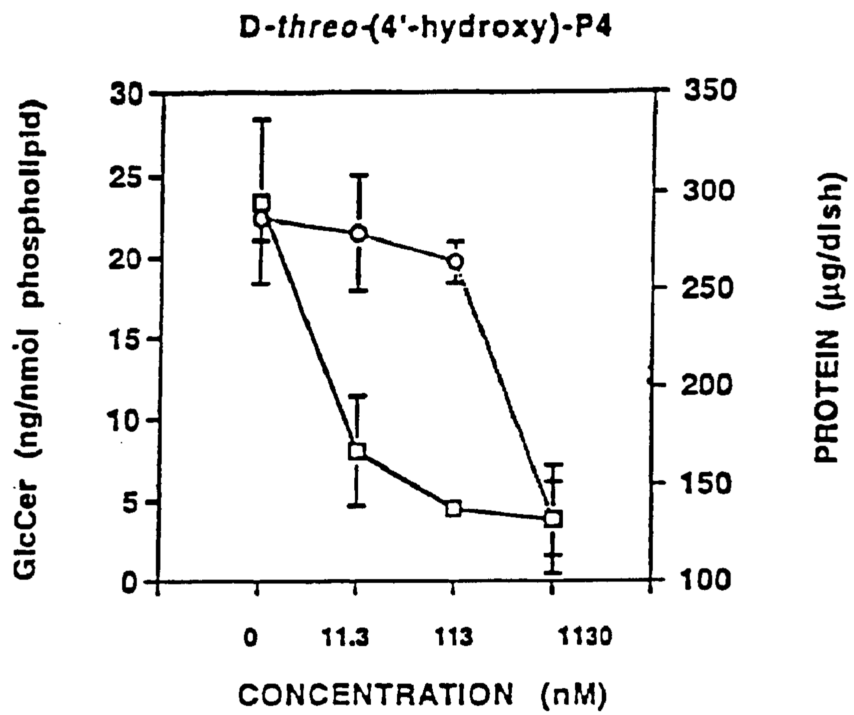


Figure 14

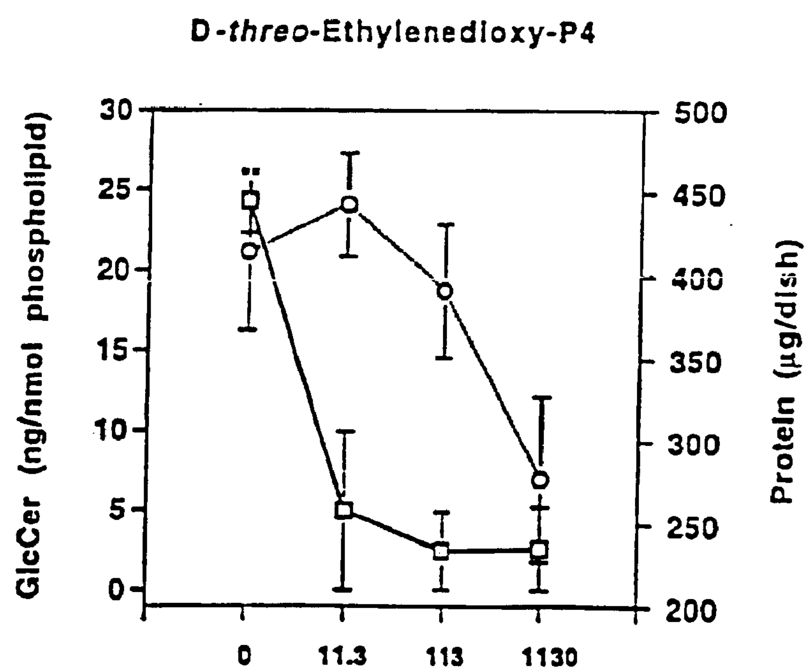


Figure 15

AMINO CERAMIDE-LIKE COMPOUNDS AND THERAPEUTIC METHODS OF USE

RELATED APPLICATIONS

This application is a continuation of U.S. app. Ser. No. 10/134,315, filed Apr. 29, 2002 now abandoned, the entire teachings of which are incorporated herein by reference.

SPONSORSHIP

The present invention was supported by grant nos. R01 DK41487, R01 DK69255 and RO139255 from the National Institutes of Health, contract R43 CA 58159 from the National Cancer Institute, grant GM 35712 from the National Institute of General Medical Sciences, and by the University of Michigan Comprehensive Cancer Center grant 2P30 CA 46592 from the National Cancer Institute, U.S. Public Health Service, DHHS. Grant number for Merit Award from Veteran's Administration. The government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates generally to ceramide-like compounds and, more particularly, to ceramide-like compounds containing a tertiary amine group and their use in therapeutic methods.

BACKGROUND OF THE INVENTION

Hundreds of glycosphingolipids (GSLs) are derived from glucosylcersmide (GlcCer), which is enzymatically formed from ceramide and UDP-glucose. The enzyme involved in GlcCer formation is UDP-glucose:N-acylsphingosine glucosyltransferase (GlcCer synthase). The rate of GlcCer formation under physiological conditions may depend on the tissue level of UDP-glucose, which in turn depends on the level of glucose in a particular tissue (Zador, I. Z. et al., "A Role for Glycosphingolipid Accumulation in the Renal Hypertrophy of Streptozotocin-Induced Diabetes Mellitus," *J. Clin. Invest.,* 91:797–803 (1993)). In vitro assays based on endogenous ceramide yield lower synthetic rates than mixtures containing added ceramide, suggesting that tissue levels of ceramide are also normally rate-limiting (Brenkert, A. et al., "Synthesis of Galactosyl Ceramide and Glucosyl Ceramide by Rat Brain: Assay Procedures and Changes with Age," *Brain Res.,* 36:183–193 (1972)).

It has been found that the level of GSLs controls a variety of cell functions, such as growth, differentiation, adhesion between cells or between cells and matrix proteins, binding of microorganisms and viruses to cells, and metastasis of tumor cells. In addition, the GlcCer precursor, ceramide, may cause differentiation or inhibition of cell growth (Bielawska, A. et al., "Modulation of Cell Growth and Differentiation by Ceramide," *FEBS Letters,* 307:211–214 (1992)) and be involved in the functioning of vitamin D_3, tumor necrosis factor-α, interleukins, and apoptosis (programmed cell death). The sphingols (sphingoid bases), precursors of ceramide, and products of ceramide catabolism, have also been shown to influence many cell systems, possibly by inhibiting protein kinase C (PKC).

It is likely that all the GSLs undergo catabolic hydrolysis, so any blockage in the GlcCer synthase should ultimately lead to depletion of the GSLs and profound changes in the functioning of a cell or organism. An inhibitor of GlcCer synthase, PDMP (1R-phenyl-2R-decanoylamino-3-morpholino-1-propanol), previously identified as the D-threo isomer (Inokuchi, J. et al., "Preparation of the

Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.,* 28:565–571 (1987)), has been found to produce a variety of chemical and physiological changes in cells and animals (Radin, N. S. et al., "Use of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol (PDMP), an Inhibitor of Glucosylceramide Synthesis," *In NeuroProtocols, A Companion to Methods in Neurosciences*, S. K. Fisher et al., Ed., (Academic Press, San Diego) 3:145–155 (1993) and Radin, N. S. et al., "Metabolic Effects of Inhibiting Glucosylceramide Synthesis with PDMP and Other Substances," *In Advances in Lipid Research; Sphingolipids in Signaling, Part B*., R. M. Bell et al., Ed. (Academic Press, San Diego) 28:183–213 (1993)). Particularly interesting is the compound's ability to cure mice of cancer induced by Ehrlich ascites carcinoma cells (Inokuchi, J. et al., "Antitumor Activity in Mice of an Inhibitor of Glycosphingolipid Biosynthesis," *Cancer Lett.,* 38:23–30 (1987)), to produce accumulation of sphingosine and N,N-dimethylsphingosine (Felding-Habermann, B. et al., "A Ceramide Analog Inhibits T Cell Proliferative Response Through Inhibition of Glycosphingolipid Synthesis and Enhancement of N,N-Dimethylsphingosine Synthesis," *Biochemistry,* 29:6314–6322 (1990)), and to slow cell growth (Shayman, J. A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.* 266:22968–22974 (1991)). Compounds with longer chain fatty acyl groups have been found to be substantially more effective (Abe, A. et al., "Improved Inhibitors of Glucosylceramide Synthesis," *J. Biochem.,* 111:191–196 (1992)).

The importance of GSL metabolism is underscored by the seriousness of disorders resulting from defects in GSL metabolizing enzymes (which diseases may collectively be referred to as "glycosphingolipidoses"). For example, Tay-Sachs, Gaucher's, and Fabry's diseases, resulting from enzymatic defects in the GSL degradative pathway and the accumulation of GSL in the patient, all have severe clinical manifestations. Another example of the importance of GSL function is seen in a mechanism by which blood cells, whose surfaces contain selecting, can, under certain conditions, bind to GSLs in the blood vessel walls and produce acute, life-threatening inflammation (Alon, R. et al., "Glycolipid Ligands for Selectins Support Leukocyte Tethering & Rolling Under Physiologic Flow Conditions," *J. Immunol.,* 154:5356–5366 (1995)).

At present there is only one treatment available for patients with Gaucher disease, wherein the normal enzyme which has been isolated from normal human tissues or cultured cells is administered to the patient. As with any drug isolated from human material, great care is needed to prevent contamination with a virus or other dangerous substances. Treatment for an individual patient is extremely expensive, costing hundreds of thousands, or even millions of dollars, over a patient's lifetime. It would thus be desirable to provide a treatment which includes administration of a compound that is readily available and/or producible from common materials by simple reactions.

Possibly of even greater clinical relevance is the role of glucolipids in cancer. For example, it has been found that certain GSLs occur only in tumors; certain GSLs occur at abnormally high concentrations in tumors; certain GSLs, added to tumor cells in culture media, exert marked stimulatory or inhibitory actions on tumor growth; antibodies to certain GSLs inhibit the growth of tumors; the GSLs that are shed by tumors into the surrounding extracellular fluid

inhibit the body's normal immunodefense system; the composition of a tumor's GSLs changes as the tumors become increasingly malignant; and, in certain kinds of cancer, the level of a GSL circulating in the blood gives useful information regarding the patient's response to treatment. Because of the significant impact GSLs have on several biochemical processes, there remains a need for compounds having improved GlcCer synthase inhibition activity.

It would thus be desirable to provide compounds which inhibit GlcCer synthase activity, thereby lowering the level of GSLs and increasing GSL precursor levels, e.g. increasing the levels of ceramide and sphingols. It would further be desirable to provide compounds which inhibit GlcCer synthase activity and lower the level of GSLs without also increasing ceramide levels. It would also be desirable to provide compounds and therapeutic methods to treat conditions and diseases associated with altered GSL levels and/or GSL precursor levels.

SUMMARY OF THE INVENTION

Novel compounds are provided which inhibit GlcCer formation by inhibiting the enzyme GlcCer synthase, thereby lowering the level of GSLs. The compounds of the present invention have improved GlcCer synthase inhibition activity and are, therefore, highly useful in therapeutic methods for treating various conditions and diseases associated with altered GSL levels, as well as GSL precursor levels. For example, the compounds of the present invention may be useful in methods involving cancer growth and metastasis, the growth of normal tissues, the ability of pathogenic microorganisms to bind to normal cells, the binding between similar cells, the binding of toxins to human cells, and the ability of cancer cells to block the normal process of immunological cytotoxic attack.

Additional objects, advantages, and features of the present invention will become apparent from the following description and appended claims, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and subjoined claims and by referencing the following drawings in which:

FIG. **1** is a graph showing the growth and survival of 9L gliosarcoma cells grown in medium containing different GlcCer synthase inhibitors;

FIG. **2** is a graph showing the protein content of MDCK cells cultured for 24 hr in medium containing different concentrations of the separated erythro- and threo-isomers of a preferred compound of the present invention;

FIG. **3** is a graph showing [^{3}H]thymidine incorporation into the DNA of MDCK cells treated with a preferred compound of the present invention;

FIGS. **4**A and **4**B are graphs showing the effects of P4 and p-methoxy-P4 on GlcCer synthase activity;

FIG. **5** is a graph showing the linear relationship between the inhibition of GlcCer synthase activity and electronic parameter (δ) and hydrophobic parameter (π);

FIG. **6** is a graph showing the effects of dioxy P4 derivatives on GlcCer synthase activity;

FIG. **7** is a bar graph showing the effects of D-t-3',4'-ethylenedioxy-P4 on GlcCer synthesis and cell growth;

FIG. **8** is a schematic of the synthetic pathway for 4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol;

FIG. **9** is an illustration of the structures of P4 and of phenyl-substituted P4 homologues;

FIG. **10** is an HPLC chromatogram showing the separation of the enantiomers of P4 and p-methoxy-P4 by chiral chromatography;

FIG. **11** is a graph showing the effects of D-threo-4'-hydroxy-P4 as compared to D-threo-p-methoxy-P4 on GlcCer synthase activity;

FIG. **12** is a graph showing the effects of D-threo enantiomers of P4, 4'-hydroxy-P4 and 3',4'-ethylenedioxy-P4 on 1-O-acyceramide synthase activity;

FIG. **13** is a graph showing the effect of D-threo-P4 on GlcCer synthesis and cell growth;

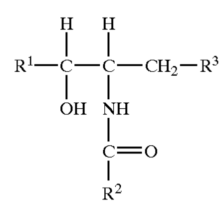
FIG. **14** is a graph showing the effect of D-threo-4'-hydroxy-P4 on GlcCer synthesis and cell growth; and

FIG. **15** is a graph showing the effect of D-threo-3',4'-ethylenedioxy-P4 on GlcCer synthesis and cell growth.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Novel compounds are provided which inhibit GlcCer formation by inhibiting the enzyme GlcCer synthase, thereby lowering the level of GSLs. The compounds of the present invention have improved GlcCer synthase inhibitory activity and are, therefore, highly useful in therapeutic methods for treating various conditions and diseases associated with altered GSL levels.

The compounds of the present invention generally have the following formula:



wherein

R^1 is a phenyl group, preferably a substituted phenyl group such as p-methoxy, hydroxy, dioxane substitutions such as methylenedioxy, ethylenedioxy, and trimethylenedioxy, cyclohexyl or other acyclic group, t-butyl or other branched aliphatic group, or a long alkyl or alkenyl chain, preferably 7 to 15 carbons long with a double bond next to the kernel of the structure. The aliphatic chain can have a hydroxyl group near the two asymmetric centers, corresponding to phytosphingosine.

R^2 is an alkyl residue of a fatty acid, 2 to 18 carbons long. The fatty acid can be saturated or unsaturated, or possess a small substitution at the C-2 position (e.g., a hydroxyl group). It is contemplated that the R^2 group fatty acid may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 carbons long. Longer fatty acids also may be useful. Preferably R^2 in the above structure is either 5 carbons or 7 carbons in length.

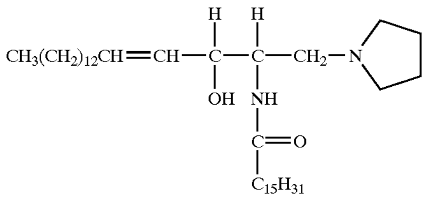
R^3 is a tertiary amine, preferably a cyclic amine such as pyrrolidine, azetidine, morpholine or piperidine, in which the nitrogen atom is attached to the kernel (i.e., a tertiary amine).

All four structural isomers of the compounds are contemplated within the present invention and may be used either singly or in combination (i.e., DL-threo or DL-erythro).

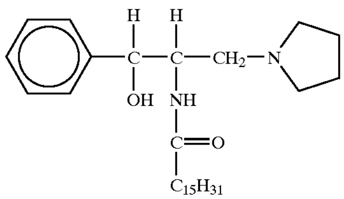
The preferred aliphatic compound of the present invention is D-threo-1-pyrrolidino-1-deoxyceramide, identified as

IV-231B herein and also referred to as PD. The preferred aromatic compound of the present invention is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, identified as BML-119 herein and also referred to as P4. The structures of the preferred compounds are as follows:

PD



P4



Additional preferred compounds of the present invention are D-t-3',4'-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, also referred to herein as D-t-3',4'-ethylenedioxy-P4, and D-t-4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, also referred to herein as D-t-4'-hydroxy-P4.

By increasing the acyl chain length of PDMP from 10 to 16 carbon atoms, the efficacy of the compounds of the present invention as GlcCer synthase inhibitors is greatly enhanced. The use of a less polar cyclic amine, especially a pyrrolidine instead of a morpholine ring, also increases the efficacy of the compounds. In addition, replacement of the phenyl ring by a chain corresponding to sphingosine yields a strongly inhibitory material. By using a chiral synthetic route, it was discovered that the isomers active against GlcCer synthase had the R,R-(D-threo)-configuration. However, strong inhibition of the growth of human cancer cells in plastico was produced by both the threo and erythro racemic compounds, showing involvement of an additional factor beyond simple depletion of cell glycosphingolipids by blockage of GlcCer synthesis. The growth arresting effects could be correlated with increases in cellular ceramide and diglyceride levels.

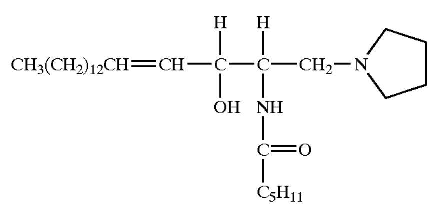
Surprisingly, the aliphatic pyrrolidino compound of the present invention (identified as IV-231B), was strongly inhibitory toward the GlcCer synthase and produced almost complete depletion of glycolipids, but did not inhibit growth or cause an accumulation of ceramide. Attempts were made to determine if the differences in growth effects could be attributed to the influence of the inhibitors on related enzymes (ceramide and sphingomyelin synthase and ceramidase and sphingomyelinase). While some stimulation or inhibition of enzyme activity was noted, particularly at high inhibitor concentrations (50 μM), these findings did not explain the differing effects of the different inhibitors.

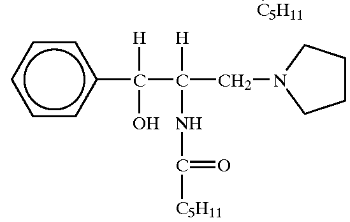
By slowing the synthesis of GlcCer, the compounds of the present invention lower the levels of all the GlcCer-derived GSLs due to the GSL hydrolases which normally destroy them. While the body will continue to make the more complex GSLs from available GlcCer, the rate of synthesis will slow down as the level of GlcCer diminishes. The rate of lowering depends on the normal rate of destruction of each GSL. These rates, however, are relatively rapid in animals and cultured cells.

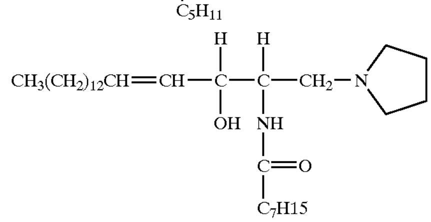
At higher dosages, many of the compounds of the present invention produce an elevation in the level of ceramide. Presumably this occurs because cells continue to make ceramide despite their inability to utilize it for GlcCer synthesis. Ceramide is also normally converted to sphingomyelin, but this process does not seem to be able to handle the excess ceramide. It has been unexpectedly found, however, that an additional process is also involved, since even those isomers that are inert against GlcCer synthase also produce an elevation in ceramide levels. Moreover, the blockage of GlcCer synthase can occur at low inhibitor dosages, yet ceramide accumulation is not produced. The preferred aliphatic compound of the present invention, D-threo-1-pyrrolidino-1-deoxyceramide (PD), does not produce ceramide accumulation at all, despite almost complete blockage of GlcCer synthesis.

This distinction between the aromatic and the aliphatic compounds of the present invention is important because ceramide has recently been proposed to cause cell death (apoptosis) by some still unknown mechanism. At lower dose levels, the aromatic compounds of the present invention cause GSL disappearance with only small accumulation of ceramide and inhibition of cell growth. Higher dosages cause much more ceramide deposition and very slow cell growth or cell death.

In certain embodiments, the inventors found that compounds containing a 16 carbon fatty acyl group is an extremely efficient and potent GlcCer synthase inhibitor. However, the longer the acyl chain of the PDMP-based compounds, the more lipophilic the agent. The inventors found that the C16 fatty acyl PDMP derivatives had a long retention time within the body. In some instances, it may be desirable to produce compounds having a C6 or C8 fatty acyl chain (i.e., R^2 in the above structures is a C5 or C7 fatty acyl chain backbone). Specifically contemplated by the present invention are compounds of the following formulas:







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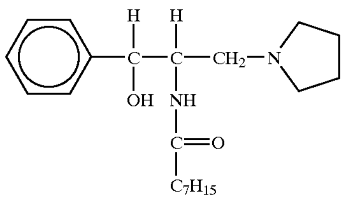


In one embodiment of the present invention, methods of treating patients suffering from inborn genetic errors in the metabolism of GlcCer and its normal anabolic products (lactosylceramide and the more complex GSLs) are provided. The presently known disorders in this category include Gaucher, Fabry, Tay-Sachs, Sandhoff, and GM1 gangliosidosis. The genetic errors lie in the patient's inability to synthesize a hydrolytic enzyme having normal efficiency. Their inefficient hydrolase allows the GSL to gradually accumulate to a toxic degree, debilitating or killing the victim. The compounds of the present invention slow the formation of GSLs, thus allowing the defective hydrolase to gradually "catch up" and restore the concentrations of GSLs to their normal levels and thus the compounds may be administered to treat such patients.

With respect to Gaucher disease, it has been calculated that much of the patient's accumulated GlcCer in liver and spleen arises from the blood cells, which are ultimately destroyed in these organs after they have reached the end of their life span. The actual fraction, lipid derived from blood cells versus lipid formed in the liver and spleen cells, is actually quite uncertain, but the external source must be important. Therefore, it is necessary for the compounds of the present invention to deplete the blood cells as they are formed or (in the case of white blood cells) while they still circulate in the blood. Judging from toxicity tests, the white cells continue to function adequately despite their loss of GSLs. Although the toxicity studies were not of a long enough duration to produce many new red cells with low GSL content, it is possible that circulating red cells also undergo turnover (continual loss plus replacement) of GSLs.

In an alternative embodiment of the present invention, for the treatment of disorders involving cell growth and division, high dosages of the compounds of the present invention are administered but only for a relatively short time. These disorders include cancer, collagen vascular diseases, atherosclerosis, and the renal hypertrophy of diabetic patients. Accumulation or changes in the cellular levels of GSLs have been implicated in these disorders and blocking GSL biosynthesis would allow the normal restorative mechanisms of the body to resolve the imbalance.

With atherosclerosis, it has been shown that arterial epithelial cells grow faster in the presence of a GlcCer product (lactosylceramide). Oxidized serum lipoprotein, a material that normally circulates in the blood, stimulates the formation of plaques and lactosylceramide in the inner lining of blood vessels. Treatment with the compounds of the present invention would inhibit this mitogenic effect.

In an additional embodiment of the present invention, patients suffering from infections may be treated with the compounds of the present invention. Many types of pathogenic bacteria have to bind to specific GSLs before they can induce their toxic effects. As shown in Svensson, M. et al., "Epithelial Glucosphingolipid Expression as a Determinant of Bacterial Adherence and Cytokine Production," *Infect. and Immun.,* 62:4404–4410 (1994), expressly incorporated by reference, PDMP treatment reduces the adherence of *E. coli* to mammalian cells. Several viruses, such as influenza type A, also must bind to a GSL. Several bacterial toxins, such as the verotoxins, cannot themselves act without first binding to a GSL. Thus, by lowering the level of GSLs, the degree of infection may be ameliorated. In addition, when a patient is already infected to a recognizable, diagnosable degree, the compounds of the present invention may slow the further development of the infection by eliminating the binding sites that remain free.

It has been shown that tumors produce substances, namely gangliosides, a family of GSLs, that prevent the host i.e., patient, from generating antibodies against the tumor. By blocking the tumor's ability to secrete these substances, antibodies against the tumor can be produced. Thus, by administering the GlcCer synthase inhibitors of the present invention to the patient, the tumors will become depleted of their GSLs and the body's normal immunological defenses will come into action and destroy the tumor. This technique was described in Inokuchi, J. et al., "Antitumor Activity in Mice of an Inhibitor of Glycosphingolipid Biosynthesis," *Cancer Lett.,* 38:23–30(1987), expressly incorporated by reference. The compounds of the present invention and in particular the aliphatic compounds require much lower doses than those previously described. This is particularly important because the lower dose may reduce certain side effects. Moreover, because the aliphatic compounds of the present invention do not produce ceramide accumulation, they are less toxic. In addition, 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4), may act via two pathways, GSL depletion and ceramide accumulation.

In an alternative embodiment, a vaccine-like preparation is provided. Here, cancer cells are removed from the patient (preferably as completely as possible), and the cells are grown in culture in order to obtain a large number of the cancer cells. The cells are then exposed to the inhibitor for a time sufficient to deplete the cells of their GSLs (generally 1 to 5 days) and are reinjected into the patient. These reinjected cells act like antigens and are destroyed by the patient's immunodefense system. The remaining cancer cells (which could not be physically removed) will also be attacked by the patient's antibodies. In a preferred embodiment, the patient's circulating gangliosides in the plasma are removed by plasmapheresis, since the circulating gangliosides would tend to block the immunodefense system.

It is believed that tumors are particularly dependent on GSL synthesis for maintenance of their growth (Hakomori, S. "New Directions in Cancer Therapy Based on Aberrant Expression of Glycosphingolipids: Anti-adhesion and Ortho-Signaling Therapy," Cancer Cells 3:461–470 (1991)). Accumulation of ceramide in treated tumors also slows their growth or kills them. Tumors also generate large amounts of GSLs and secrete them into the patient's body, thereby preventing the host's normal response by immunoprotective cells, which should generate antibodies against or otherwise destroy tumor cells (e.g., tumors are weakly antigenic). It has also been shown that GSL depletion blocks the metastasis of tumor cells (Inokuchi, J. et al., "Inhibition of Experimental Metastasis of Murine Lewis Long Carcinoma by an Inhibitor of Glucosylceramide Synthase and its Possible Mechanism of Action," *Cancer Res.,* 50:6731–6737 (1990). Tumor angiogenesis (e.g., the production of blood capillaries) is strongly influenced by GSLs (Ziche, M. et al., "Angiogenesis Can Be Stimulated or Repressed in In Vivo by a Change in GM3:GD3 Ganglioside Ratio," *Lab. Invest.,* 67:711–715 (1992)). Depleting the tumor of its GSLs should block the tumors from generating the new blood vessels they need for growth.

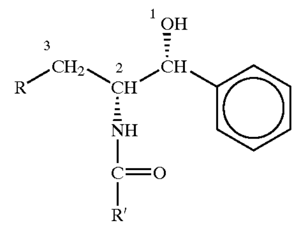
A further important characteristic of the compounds of the present invention is their unique ability to block the growth of multidrug resistant ("MDR") tumor cells even at much lower dosages. This was demonstrated with PDMP by Rosenwald, A. G. et al., "Effects of the Glycosphingolipid Synthesis Inhibitor, PDMP, on Lysosomes in Cultured Cells," *J. Lipid Res.,* 35:1232 (1994), expressly incorporated by reference. Tumor cells that survive an initial series of therapeutic treatments often reappear some years later with new properties—they are now resistant to a second treatment schedule, even with different drugs. This change has been attributed to the appearance in the tumor of large amounts of a specific MDR protein (P-glycoprotein). It has been suggested that protein kinase C (PKC) may be involved in the action or formation of P-glycoprotein (Blobe, G. C. et al., "Regulation of PKC and Its Role in Cancer Biology," *Cancer Metastasis Rev.,* 13:411–431 (1994)). However, decreases in PKC have other important effects, particularly slowing of growth. It is known that PDMP does lower the cellular content of PKC (Shayman, J. A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.,* 266:22968–22974 (1991)) but it is not clear why it so effectively blocks growth of MDR cells (Rosenwald, A. G. et al., "Effects of the Glycosphingolipid Synthesis Inhibitor, PDMP, On Lysosomes in Cultured Cells," *J. Lipid Res.,* 35:1232 (1994)). A recent report showed that several lipoidal amines that block MDR action also lower the level of the enzyme acid sphingomyelinase (Jaffrezou, J. et al., "Inhibition of Lysosomal Acid Sphingomyelinase by Agents which Reverse Multidrug Resistance," *Biochim. Biophys. Acta,* 1266:1–8 (1995)). One of these agents was also found to increase the cellular content of sphingosine 5-fold, an effect seen with PDMP as well. One agent, chlorpromazine, behaves like the compounds of the present invention, in its ability to lower tissue levels of GlcCer (Hospattankar, A. V. et al., "Changes in Liver Lipids After Administration of 2-Decanoylamino-3-Morpholinopropiophenone and Chlorpromazine," *Lipids,* 17:538–543 (1982)).

It will be appreciated by those skilled in the art that the compounds of the present invention can be employed in a wide variety of pharmaceutical forms; the compound can be employed neat or admixed with a pharmaceutically acceptable carrier or other excipients or additives. Generally speaking, the compound will be administered orally or intravenously. It will be appreciated that therapeutically acceptable salts of the compounds of the present invention may also be employed. The selection of dosage, rate/frequency and means of administration is well within the skill of the artisan and may be left to the judgment of the treating physician or attending veterinarian. The method of the present invention may be employed alone or in conjunction with other therapeutic regimens. It will also be appreciated that the compounds of the present invention are also useful as a research tool e.g., to further investigate GSL metabolism.

The following Specific Example further describes the compounds and methods of the present invention.

SPECIFIC EXAMPLE 1

The following formulas set forth preferred aromatic and aliphatic compounds:

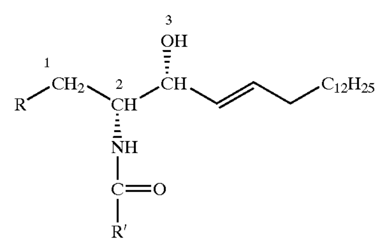


identified as (1R,2R)-1-phenyl-2-acylamino-3-cyclic amino-1-propanol, and referred to herein as the "aromatic inhibitors," wherein

The phenyl group can be a substituted phenyl group (such as p-methoxyphenyl).

R' is an alkyl residue of a fatty acid, 2 to 18 carbons long. The fatty acid can be saturated or unsaturated, or possess a small substitution at the C-2 position (e.g., a hydroxyl group). It is contemplated that the R' group fatty acid may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 carbons long. Longer fatty acids also may be useful. Preferrably R' in the above structure is either 5 carbons or 7 carbons in length.

R is morpholino, pyrrolidino, piperidino, azetidino (trimethyleneimino), N-methylethanolamino, diethylamino or N-phenylpiperazino. A small substituent, such as a hydroxyl group, is preferably included on the cyclic amine moiety.



identified as (2R,3R)-2-palmitoyl-sphingosyl amine or 1-cyclic amino-1-deoxyceramide or 1-cyclic amino-2-hexadecanoylamino-3-hydroxy-octadec-4,5-ene, and referred to herein as the "aliphatic inhibitors," wherein

R' is an alkyl residue of a fatty acid, 2 to 18 carbons long. The fatty acid can be saturated or unsaturated, or possess a small substitution at the C-2 position (e.g., a hydroxyl group). It is contemplated that the R' group fatty acid may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 carbons long. Longer fatty acids also may be useful. Preferrably R' in the above structure is either 5 carbons or 7 carbons in length.

R is morpholino, pyrrolidino, piperidino, azetidino (trimethyleneimino), N-methylethanolamino, diethylamino or N-phenylpiperazino. A small substituent, such as a hydroxyl group, is preferably included on the cyclic amine moiety.

The long alkyl chain shown in Formula II can be 8 to 18 carbon atoms long, with or without a double bond near the asymmetric carbon atom (carbon 3). Hydroxyl groups can, with advantage, be substituted along the aliphatic chain, particularly on carbon 4 (as in the naturally occurring sphingol, phytosphingosine). The long chain can also be replaced by other aliphatic groups, such at t-butyl or cyclopentyl.

The aromatic inhibitors (see Formula I and Table 1) were synthesized by the Mannich reaction from 2-N-

acylaminoacetophenone, paraformaldehyde, and a secondary amine as previously described (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.,* 28:565–571 (1987) and Vunnam, R. R. et al., "Analogs of Ceramide that Inhibit Glucocerebroside Synthetase in Mouse Brain," *Chem. Phys. Lipids,* 26:265–278 (1980)). For those syntheses in which phenyl-substituted starting materials were used, the methyl group in the acetophenone structure was brominated and converted to the primary amine. Bromination of p-methoxyacetophenone was performed in methanol. The acetophenones and amines were from Aldrich Chemical Co., St. Louis, Mo. Miscellaneous reagents were from Sigma Chemical Co. and the sphingolipids used as substrates or standards were prepared by methods known in the art. The reactions produce a mixture of four isomers, due to the presence of two asymmetric centers.

The aliphatic inhibitors (See Formula II and Table 2) were synthesized from the corresponding 3-t-butyldimethylsilyl-protected sphingols, prepared by enantioselective aldol condensation (Evans, D. A. et al., "Stereoselective Aldol Condensations Via Boron Enolates," *J. Am. Chem. Soc.,* 103:3099–3111 (1981) and Abdel-Magid, A. et al., "Metal-Assisted Aldol Condensation of Chiral A-Halogenated Imide Enolates: A Stereocontrolled Chiral Epoxide Synthesis," *J. Am. Chem. Soc.,* 108:4595–4602 (1986)) using a modification of the procedure of Nicolaou et al. (Nicolaou, K. C. et al., "A Practical and Enantioselective Synthesis of Glycosphingolipids and Related Compounds. Total Synthesis of Globotriaosylceramide (Gb_3)," *J. Am. Chem. Soc.,* 110:7910–7912 (1988)). Each protected sphingol was first converted to the corresponding primary triflate ester, then reacted with a cyclic amine. Subsequent N-acylation and desilylation led to the final products in good overall yield (Carson, K. G. et al., "Studies on Morpholinosphingolipids: Potent Inhibitors of Glucosylceramide Synthase," *Tetrahedron Lett.,* 35:2659–2662 (1994)). The compounds can be called 1-morpholino-(or pyrrolidino)-1-deoxyceramides.

Labeled ceramide, decanoyl sphingosine, was prepared by reaction of the acid chloride and sphingosine (Kopaczyk, K. C. et al., "In Vivo Conversions of Cerebroside and Ceramide in Rat Brain," *J. Lipid Res.,* 6:140–145 (1965)) and NBD-SM (12-[N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]-sphingosylphosphorylcholine) was from Molecular Probes, Inc., Eugene, Oreg.

Methods

TLC of the amines was carried out with HPTLC plates (E. Merck silica gel 60) and C-M-HOAc 90:10:10 (solvent A) or 85:15:10 (solvent B) or C-M-conc. ammonium hydroxide 30:10:1 (solvent C). The bands were stained with iodine or with Coomassie Brilliant Blue R-250 (Nakamura, K. et al., "Coomassie Brilliant Blue Staining of Lipids on Thin-Layer Plates," *Anal. Biochem.,* 142:406–41 (1984)) and, in the latter case, quantified with a Bio-Rad Model 620 videodensitometer operated with reflected white light. The faster band of each PDMP analog, previously identified as the erythro form, corresponds to the 1S,2R and 1R,2S stereoisomers, and the slower band, previously identified as the threo form, corresponds to the 1R,2R and 1S,2S stereoisomers.

TLC of the cell lipids was run with C-M-W 24:7:1 (solvent D) or 60:35:8 (solvent E).

Growth of cell lines. Comparisons of different inhibitors with regard to suppression of human cancer cell growth were made by the University of Michigan Cancer Center in vitro Drug Evaluation Core Laboratory. MCF-7 breast carcinoma cells, HT-29 colon adenocarcinoma cells, H-460 lung large cell carcinoma cells, and 9L brain gliosarcoma cells were grown in RPMI 1640 medium with 5% fetal bovine serum, 2 mM glutamine, 50 units/ml of penicillin, 50 mg/ml of streptomycin, and 0.1 mg/ml of neomycin. UMSCC-10A head and neck squamous carcinoma cells were grown in minimal essential medium with Earle salts and the same supplements. Medium components were from Sigma Chemical Co. Cells were plated in 96-well microtiter plates (1000 cells/well for H-460 and 9L cells, and 2000 cells/well for the other lines), and the test compounds were added 1 day later. The stock inhibitor solutions, 2 mM in 2 mM BSA, were diluted with different amounts of additional 2 mM BSA, then each solution was diluted 500-fold with growth medium to obtain the final concentrations indicated in the Figures and Tables.

Five days after plating the H-460 and 9L cells, or 6 days for the other lines, cell growth was evaluated by staining the adhering cells with sulforhodamine B and measuring the absorbance at 520 nm (Skehan, P. et al., "New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening," *J. Natl. Cancer Inst.,* 82:1107–1112 (1990)). The absorbance of the treated cultures is reported as percent of that of control cultures, to provide an estimate of the fraction of the cells that survived, or of inhibition of growth rate.

For the experiments with labeled thymidine, each 8.5 cm dish contained 500,000 Madin-Darby canine kidney (MDCK) cells in 8 ml of Dulbecco modified essential supplemented medium. The cells were incubated at 37° C. in 5% CO_2 for 24 h, then incubated another 24 h with medium containing the inhibitor-BSA complex. The control cells were also incubated in the presence of BSA. The cells were washed with phosphate/saline and trichloroacetic acid, then scraped off the dishes, dissolved in alkali, and analyzed for protein and DNA incorporated tritium. [Methyl-^{3}H] thymidine (10 μCi) was added 4 h prior to harvesting.

Assay of sphingolipid enzymes. The inhibitors were evaluated for their effectiveness against the GlcCer synthase of MDCK cell homogenates by incubation in a thermostatted ultrasonic bath (Radin N. S. et al., "Ultrasonic Baths as Substitutes for Shaking Incubator Baths," *Enzyme,* 45:67–70 (1991)) with octanoyl sphingosine and uridinediphospho [^{3}H]glucose (Shukla, G. S. et al., "Glucosylceramide Synthase of Mouse Kidney: Further Characterization and Improved Assay Method," *Arch. Biochem. Biophys.,* 283:372–378 (1990)). The lipoidal substrate (85 μg) was added in liposomes made from 0.57 mg dioleoylphosphatidylcholine and 0.1 mg of Na sulfatide. Confluent cells were washed, then homogenized with a micro-tip sonicator at 0° C. for 3×30 sec; ~0.2 mg of protein was used in each assay tube. In the case of the aromatic inhibitors, the test compound was simply evaporated to dryness from solution in the incubation tube. This method of adding the inhibitor was found to give the same results as addition as a part of the substrate liposomes. The aliphatic inhibitors, which appeared to be less soluble in water, were added as part of the substrate liposomes.

Acid and neutral ceramidases were assayed under conditions like those above, but the medium contained 110 μM [1-^{14}C]decanoyl sphingosine (10^5 cpm) in 340 μM dioleoylphosphatidylcholine liposomes and 0.34 mg of MDCK cellular protein homogenate. The acid enzyme was incubated in 32.5 mM citrate-Na^+ (pH 4.5) and the neutral enzyme buffer was 40 mM Tris-Cl^- (pH 7.1 at 37° C.). After 60 min in the ultrasonic bath, 3 ml of C-M 2:1, carrier decanoic acid, and 0.6 ml of 0.9% saline were added and the

lipids in the lower layer were separated by TLC with C-HOAc 9:1. The liberated decanoic acid was scraped off the glass plate and counted.

Ceramide synthase was assayed with 1 μM [3-^{3}H] sphingosine (70,000 cpm, repurified by column chromatography), 0.2 mM stearoyl-CoA, 0.5 mM dithiothreitol, and ~300 μg of MDCK homogenate protein in 25 mM phosphate-K^+ buffer, pH 7.4, in a total volume of 0.2 ml. The incubation (for 30 min) and TLC were carried out as above and the ceramide band was counted.

Sphingomyelin synthase was evaluated with 44 μM [^{14}C] decanoyl sphingosine (10^5 cpm) dispersed with 136 μM dioleoyllecithin as in the ceramide synthase assay, and 5 mM EDTA and 50 mM Hepes-Na^+ pH 7.5, in a total volume of 0.5 ml. MDCK homogenate was centrifuged at 600×g briefly, then at 100,000×g for 1 h, and the pellet was suspended in water and sonicated with a dipping probe. A portion of this suspension containing 300 μg of protein was used. Incubation was at 37° C. for 30 min, after which the lipids were treated as above, using C-M-W 60:35:8 for the isolation of the labeled decanoyl SM.

Acid and neutral SMase assays were based on the procedures of Gatt et al. (Gatt, S. et al., "Assay of Enzymes of Lipid Metabolism With Colored and Fluorescent Derivatives of Natural Lipids," *Meth. Enzymol.,* 72:351–375 (1981)), using liposomes containing NBD-SM dispersed like the labeled ceramide (10 μM substrate and 30 μM lecithin). The assay medium for the neutral enzyme also contained 50 mM Tris-Cl^- (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$ and 0.29 mg of MDCK cell protein in a total volume of 0.25 ml. Incubation was at 37° C. for 30 min in the ultrasonic bath, then the fluorescent product, NBD-ceramide, was isolated by partitioning the assay mixture with 0.45 ml 2-propanol, 1.5 ml heptane, and 0.2 ml water. After centrifugation, a trace of contaminating NBD-SM was removed from 0.9 ml of the upper layer by washing with 0.35 ml water. The upper layer was analyzed with a fluorometer (460 nm excitation, 515 nm emission).

Acid SMase was assayed with the same liposomes in 0.2 ml of assay mixture containing 125 mM NaOAc (pH 5.0) and 61 μg of cell protein, with 60 min of incubation at 37° C. The resultant ceramide was determined as above.

Results

Table 1 lists the aromatic compounds (see Formula I) synthesized and their migration rates on silica gel TLC plates. Separation of the threo- and erythro-steroisomers by TLC was generally very good, except for BML-120, -121, and -122 in the acidic solvent. In the basic solvent BML-119 and BML-122 yielded poorly resolved double bands. BML-112 was unexpectedly fast-running, especially when compared with BML-120; both are presumably dihydrochlorides.

TABLE 1

STRUCTURES OF THE AROMATIC INHIBITORS

BML Number or Name	R Group	Phenyl Substituent	TLC hR_1 Value[a]
PDMP[b]	morpholino		34(47)
PPMP	morpholino		(53)
112	N-phenylpiperazino		56
113	morpholino	p-fluoro	25
114	diethylamino		25
115	piperidino (pentamethyleneimino)		29
116	hexamethyleneimino		34
117[b]	morpholino	p-fluoro	41
118	piperidino	p-fluoro	26
119	pyrrolidino (tetramethyleneimino)		20–70(44)
120	1-methylpiperazino		7–62
121	3-dimethylaminopiperidino		1–30
122	N-methylethanolamino		6–71
123	azetidino (trimethyleneimino)		12
124	amino		15
125	morpholino	p-methoxy	37
126	pyrrolidino	p-methoxy	(50)

[a]Only the relative R_f value of the faster-moving band is shown. The first value was obtained with solvent A, the second with solvent C, and the numbers in parentheses, with solvent B. In the case of BML-117, -125, and -126, a 20-cm high TLC plate was used to improve the separation.
[b]The fatty acid chain suggested by the R' group is decanoyl, not palmitoyl.

Table 2 describes four aliphatic inhibitors (see Formula II), which can be considered to be ceramide analogs in which the C-1 hydroxyl group is replaced by a cyclic amine. It should be noted that the carbon frameworks of compounds in Tables 1 and 2 are numbered differently (see Formulas I and II), thus affecting comparisons of stereochemical configurations. The threo- and erythro-isomers separated very poorly on TLC plates. Like the aromatic inhibitors, however, the morpholine compounds ran faster than the pyrrolidine compounds. The latter are presumably more strongly adsorbed by the silica gel because they are more basic.

TABLE 2

CHARACTERIZATION OF THE SPHINGOSYL INHIBITORS

Number	R Group	Sphingol Structure	TLC hR_f Value[a]
IV-181A	morpholino	2R, 3S	43
IV-206A	morpholino	2R, 3R	40
IV-230A	pyrrolidino	2R, 3S	31
IV-231B	pyrrolidino	2R, 3R	31

[a]TLC solvent: C-M-HOAc 90:5:10. Similar but faster migrations were obtained with solvent A.

Structure-activity correlations. The results of testing the compounds in an assay system for GlcCer synthase are listed in Table 3. Each inhibition determination (±SD) shown in Table 3 was carried out in triplicate. Some of the inhibitors were tested as mixtures of DL-erythro- and DL-threo-isomers (see column 4). Only the D-threo enantiomer in each mixture was predicted to be the actual enzyme inhibitor (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.,* 28:565–571 (1987)); the content of this isomer was calculated by measuring the proportions of the threo- and erythro-racemic mixtures by quantitative TLC. The DL-threo contents were found to be in the range of 40 to 72%. The comparisons, in the case of the mixtures, are therefore approximate (most of the samples were not purified to remove the three less-active isomers and the observed data were not corrected for the level of the primary enantiomers). The separation of the threo- and erythro-forms is most

conveniently accomplished by crystallization, but the specific conditions vary for each substance; thus only BML-119, a strong inhibitor, was separated into its threo- and erythro-forms. BML-112 is not included in Table 3 because it had no inhibitory activity against GlcCer synthase of rabbit liver microsomes.

TABLE 3

Inhibition of Ceramide Glucosyltransferase of MDCK cell Homogenates by Different Compounds

Inhibitor Number	% Inhibition at 80 μM	Inhibition at 5 μM	Active Isomer[h]
BML-113	60 ± 4.7[a]		29
BML-114	31 ± 2.9[a]		20
BML-115	84 ± 0.8[a]	12.4 ± 0.7[f]	27
	82 ± 0.3[b]		
BML-116	28 ± 3.2[a]		27
BML-117	35 ± 0.6[b]		36
BML-118	62 ± 0.4[b]	8.3 ± 1.4[f]	32
BML-119	94 ± 1.4[b]	51 ± 2.3[e]	29
	97 ± 0.1[c]	49 ± 0.8[f]	
	96 ± 0.1[d]		
BML-120	11 ± 3.0[c]		26
BML-121	11 ± 0.4[c]		28
BML-122	58 ± 1.6[d]		26
BML-123	86 ± 0.1[d]	15 ± 0.8[f]	33
BML-124	−2 ± 1.6[d]		15
BML-125		9 ± 3.0[e]	26
BML-126	60 ± 1.8[e]	54 ± 0.3[f]	34
PDMP	90 ± 0.8[a]	16 ± 1.8[f]	100
PPMP		32 ± 1.8[e]	100
		32 ± 0.7[f]	
IV-181A		12 ± 0.2[g]	100
IV-206A		73 ± 1.5[g]	100
IV-230A		19 ± 2.1[g]	100
IV-231B		87 ± 0.4[g]	100

[a–g]Different samples were assayed as parts of different experiments.
[h]Percent of the active D-stereoisomer in the synthesized sample, estimated by scanning the two stained bands, assuming the slower one was the (racemic) active form.

Comparison of PDMP (1R,2R-decanoate) and PPMP (1R, 2R-palmitate), when evaluated at the same time in Expt. f, shows that an increase in the chain length of the N-acyl group from 10 to 16 carbon atoms distinctly improved the inhibitory activity against GlcCer synthase, as noted before (Abe, A. et al., "Improved Inhibitors of Glucosylceramide Synthesis," *J. Biochem.,* 111:191–196 (1992)). Accordingly, most of the other compounds were synthesized with the palmitoyl group for comparison with PPMP. The comparisons between the best inhibitors are clearer at the 5 μM level.

Replacing the oxygen in the morpholine ring of PPMP with a methylene group (BML-115) improved activity ~1.4-fold (calculated from the inhibitions at 5 μM in Expt. f and relative purities, and assuming that the percent inhibition is proportional to concentration in this region: 12.4/27×100/32=1.4). Previous comparison with mouse brain, human placenta, and human Gaucher spleen glucosyltransferase also showed that replacing the morpholino ring with the piperidino ring in a ketone analog of PDMP (1-phenyl-2-decanoylamino-3-piperidino-1-propanone) produced a much more active inhibitor (Vunnam, R. R. et al., "Analogs of Ceramide that Inhibit Glucocerebroside Synthetase in Mouse Brain," *Chem. Phys. Lipids,* 26:265–278 (1980)).

Replacing the piperidine group with a 7-membered ring (BML-116) greatly decreased the activity, while use of a 5-membered ring (BML-119) quadrupled the effectiveness (50 vs 12.4% inhibition). A 4-membered ring (BML-123) yielded a compound about as effective as the piperidino compound. The parent amine (BML-124), its N,N-diethyl analog (BML-114), and the sterically bulky N-phenylpiperazine analog (BML-112) displayed little or no activity.

Replacing a hydrogen atom with a fluorine atom in the p-position of the phenyl ring decreased the inhibitory power (BML-117 vs PDMP and BML-118 vs BML-115). Substitution of the p-position with an electron-donating moiety, the methoxy group, had a similar weakening effect in the case of the morpholino compound (BML-125 vs PPMP). Comparison of the pyrrolidino compounds, which are more basic than the morpholino compounds, showed that the methoxy group enhanced the inhibitory power (BML-126 vs BML-119).

Preparations of BML-119 were separated into threo and erythro racemic mixtures by HPLC on a Waters Microbondapak C_{18} column, using M-W-conc. NH_4OH 90:10:0.2 as the elution solvent. The material eluting earlier (but migrating more slowly on a TLC plate) was called BML-130; the later eluting material (faster by TLC) was called BML-129. Assay of GlcCer synthase with each preparation at 5 μM showed 15% inhibition by BML-129 and 79% inhibition by BML-130. TLC analysis of the two preparations revealed incomplete separation, which could explain the minor inhibition by BML-129. When the two stereoisomers were separated by preparative TLC, the difference in effectiveness was found to be somewhat higher, evidently due to the better separation by this method. Thus, the slower-migrating stereoisomer accounted for all or nearly all of the inhibitory activity, as noted with PDMP (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.,* 28:565–571 (1987)).

Comparison of the two pairs of aliphatic inhibitors (bottom of Table 3) showed that the 2R,3R (D-threo) form is the primary inhibitor of glucosyltransferase. This finding is in agreement with previous identification of the active PDMP isomer as being the D-threo enantiomer. However, unlike the aromatic analog, BML-129 (2R,3S/2S,3R), there was a relatively small but significant activity in the case of the (erythro) 2R,3S stereoisomer. The erythro form of PDMP was found to inhibit cell proliferation of rabbit skin fibroblasts almost as well as R,R/S,S-PDMP but it did not act on the GSLs (Uemura, K. et al., "Effect of an Inhibitor of Glucosylceramide Synthesis on Cultured Rabbit Skin Fibroblasts," *J. Biochem., (Tokyo)* 108:525–530 (1990)). As noted with the aromatic analogs; the pyrrolidine ring was more effective than the morpholine ring (Table 3).

Comparison of the aliphatic and corresponding aromatic inhibitors can be made in the case of the optically active morpholine compounds PPMP and IV-206A, both of which have the R,R structure and the same fatty acid. Here it appears that the aliphatic compound is more effective (Table 3). However, in a second comparison, at lower concentrations with the inhibitors incorporated into the substrate liposomes, the degree of inhibition was 77±0.9% with 3 μM IV-231B and 89±0.6% with 6 μM DL-threo BML-119.

Evaluations of cultured cell growth. Exposure of five different cancer cell lines to inhibitors at different concentrations for 4 or 5 days showed that the six BML compounds most active against GlcCer synthase were very effective growth inhibitors (Table 4). The IC_{50} values (rounded off to one digit in the table) ranged from 0.7 to 2.6 μM.

TABLE 4

Inhibition of Tumor Cell Growth In Vitro by Various Inhibitors

CELL TYPE	BML-115	BML-118	BML-119	BML-123	BML-126	BML-129	BML-130
MCF-7	2	2	2	2	1	3	2
H-460	2	2	1	1	1	2	3
HT-29	2		1	2	1	2	2
9L	2	2	1	2	2	2	2
UMSC C-10A	1		1	1	1	2	2

FIG. **1** shows growth and survival of 9L gliosarcoma cells grown in medium containing different GlcCer synthase inhibitors, as described above. The BML compounds were used as synthesized (mixtures of DL-threo and -erythro stereoisomers) while the PDMP and PPMP were optically resolved R,R isomers. The concentrations shown are for the mixed racemic stereoisomers, since later work (Table 4) showed that both forms were very similar in effectiveness. FIG. **1** illustrates the relatively weak effectiveness of R,R-PPMP and even weaker effectiveness of R,R-PDMP. The three new compounds, however, are much better inhibitors of GlcCer synthase and growth. These differences in growth inhibitory power correlate with their effectiveness in MDCK cell homogenates as GlcCer synthase inhibitors. Some differences can be expected due to differences in sensitivity of the synthase occurring in each cell type (the synthases were assayed only in MDCK cells).

Growth inhibition by each of the most active BML compounds occurred in an unusually small range of concentrations (e.g., the slopes of the cytotoxic regions are unusually steep). Similar rapid drop-offs were seen in another series of tests with 9L cells, in which BML-119 yielded 71% of the control growth with 1 μM inhibitor, but only 3% of control growth with 3 μM. Growth was 93% of control growth with 2 μM BML-130 but only 5% of controls with 3 μM inhibitor. While some clinically useful drugs also show a narrow range of effective concentrations, this is a relatively uncommon relationship.

When the erythro- and threo-stereoisomeric forms of BML-119 (-129 and -130) were compared, they were found to have similar effects on tumor cell growth (Table 4). This observation is similar to the results with PDMP isomers in fibroblasts cited above (Uemura, K. et al., "Effect of an Inhibitor of Glucosylceramide Synthesis on Cultured Rabbit Skin Fibroblasts," *J. Biochem., (Tokyo)* 108:525–530 (1990)). Since enzymes are optically active and since stereoisomers and enantiomers of drugs can differ greatly in their effect on enzymes, it is likely that BML-129 and BML-130 work on different sites of closely related metabolic steps.

FIG. **2** shows the amount of cellular protein per dish for MDCK cells cultured for 24 h in medium containing different concentrations of the separated erythro- and threo-isomers of BML-119, as percent of the incorporation by cells in standard medium. Each point shown in FIG. **2** is the average of values from three plates, with error bars corresponding to one standard deviation.

FIG. **3** shows [^{3}H]thymidine incorporation into DNA of MDCK cells incubated as in FIG. **2**. The values in FIG. **3** are normalized on the basis of the protein content of the incubation dishes and compared to the incorporation by cells in standard medium.

FIGS. **2** and **3** thus provide comparison of the two stereoisomers with MDCK cells. The isomers were found to inhibit growth and DNA synthesis with similar effectiveness. Thus, the MDCK cells behaved like the human tumor cells with regard to IC_{50} and the narrow range of concentrations resulting in inhibition of protein and DNA synthesis.

Surprisingly, the aliphatic inhibitor IV-231B exerted no inhibitory effect on MDCK cell growth when incubated at 20 μM for 1 day or 1 μM for 3 days. Tests with a longer growth period, 5 days, in 5 μM inhibitor also showed no slowing of growth. The dishes of control cells, which contained BSA as the only additive to the medium, contained 3.31±0.19 mg of protein, while the IV-231B/BSA treated cells contained 3.30±0.04 mg.

Lipid changes induced in the cells. Examination by TLC of the alkali-stable MDCK lipids after a 24 h incubation disclosed that BML-130 was more effective than BML-129 in lowering GlcCer levels, as expected from its greater effectiveness in vitro as a glucosyltransferase inhibitor. The level of GlcCer, estimated visually, was greatly lowered by 0.3 μM BML-130 or 0.5 μM BML-129. The levels of the other lipids visible on the plate (mainly sphingomyelin (SM), cholesterol, and fatty acids) were changed little or not at all. BML-129 and the GlcCer synthase inhibitor, BML-130, were readily detected by TLC at the various levels used, showing that they were taken up by the cells during the incubation period at dose-dependent rates. Lactosylceramide overlapped the inhibitor bands with solvent D but was well separated with solvent E, which brought the inhibitors well above lactosylceramide.

Ceramide accumulation was similar for both stereoisomers (data not shown). An unexpected finding is that noticeable ceramide accumulation appeared only at inhibitor concentrations that were more than enough to bring GlcCer levels to a very low point (e.g., at 2 or 4 μM). The changes in ceramide concentration were quantitated in a separate experiment by the diglyceride kinase method, which allows one to also determine diacylglycerol (DAG) concentration (Preiss, J. E. et al., "Quantitative Measurement of SN-1,2-Diacylglycerols Present in Platelets, Hepatocytes, and Ras- and Sis-Transformed Normal Rat Kidney Cells," *J. Biol. Chem.,* 261:8597–8600 (1986)). The results (Table 5) are similar to the visually estimated ones: at 0.4 μM BML-129 or -130 there was little effect on ceramide content but at 4 μM inhibitor, a substantial increase was observed. (While the duplicate protein contents per incubation dish were somewhat erratic in the high-dose dishes, in which growth was slow, the changes were nevertheless large and clear.) Accumulation of ceramide had previously been observed with PDMP, at a somewhat higher level of inhibitor in the medium (Shayman, J. A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.,* 266:22968–22974 (1991)). From the data for cellular protein per incubation dish, it can be seen that there

was no growth inhibition at the 0.4 μM level with either compound but substantial inhibition at the 4 μM level, especially with the glucosyltransferase inhibitor, BML-130. This finding is similar to the ones made in longer incubations with human cancer cells.

TABLE 5

Effects of BML-129 and -130 on MDCK Cell Growth and the Content of Ceramide and Diacylglycerol

Growth Medium	Protein μg/dish	Ceramide nmol/mg	Diglyceride protein
Controls	490	1.04	4.52
	560	0.96	5.61
0.4 μm BML-129	500	1.29	5.51
	538	0.99	5.13
0.4 μm BML-130	544	0.94	4.73
	538	0.87	5.65
4 μm BML-129	396	3.57	9.30
	311	3.78	9.68
4 μm BML-130	160	5.41	11.9
	268	3.34	8.71

In a separate study of ceramide levels in MDCK cells, BML-130 at various concentrations was incubated with the cells for 24 h. The ceramide concentration, measured by TLC densitometry, was 1.0 nmol/mg protein at 0.5 μM, 1.1 at 1 μM, 1.5 at 2 μM, and 3.3 at 4 μM. The results with BML-129 were virtually identical.

It is interesting that the accumulation of ceramide paralleled an accumulation of diacylglycerol (DAG), as observed before with PDMP (Shayman, J. A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.*, 266:22968–22974 (1991)). DAG is ordinarily considered to be an activator of protein kinase C and thus a growth stimulator, but the low level of GlcCer in the inhibited cells may counteract the stimulatory effect. Ceramide reacts with lecithin to form SM and DAG, so it is possible that the increased level of the latter reflects enhanced synthesis of the phosphosphingolipid rather than an elevated attack on lecitin by phospholipase D. Arabinofuranosylcytosine (ara-C), an antitumor agent, also produces an elevation in the DAG and ceramide of HL-60 cells (Strum, J. C. et al., "1-β-D-Arabinofuranosylcytosine Stimulates Ceramide and Diglyceride Formation in HL-60 Cells," *J. Biol Chem.*, 269:15493–15497 (1994)).

TLC of MDCK cells grown in the presence of 0.02 to 1 μM IV-231B for 3 days showed that the inhibitor indeed penetrated the cells and that there was a great depletion of GlcCer, but no ceramide accumulation. The depletion of GlcCer was evident even at the 0.1 μM level and virtually no GlcCer was visible at the 1 μM level; however, the more polar GSLs were not affected as strongly. After incubation for 5 days in 5 μM inhibitor, all the GSLs were virtually undetectable. The ceramide concentrations in the control and depleted cells were very similar: 13.5±1.4 vs 13.9±0.2 μg/mg protein.

The lack of ceramide accumulation in cells exposed to the aliphatic inhibitors was examined further to see if it might be due to differential actions of the different inhibitors on additional enzymes involving ceramide metabolism. For example, IV-231B might block ceramide synthase and thus prevent accumulation despite the inability of the cells to utilize ceramide for GlcCer synthesis. However, assay of ceramide synthase in homogenized cells showed it was not significantly affected by 5 μM inhibitors (Table 6). There did appear to be moderate inhibition at the 50 μM level with PDMP and the aliphatic inhibitor.

TABLE 6

Effect of Inhibitors on Acid and Neutral Ceramidases and Ceramide Synthase of MDCK Cells

Inhibitor Tested	Enzyme Activity (% of control) Ceramidase pH 4.5	Ceramidase pH 7.4	Ceramide Synthase
D-threo-PDMP, 5 μM	97 ± 4	116 ± 19	99 ± 5
D-threo-PDMP, 50 μM	133 ± 13[a]	105 ± 11	66 ± 9[a]
BML-129, 5 μM	108 ± 8	100 ± 0	97 ± 0
BML-129, 50 μM	171 ± 26[a]	99 ± 2	102 ± 1
BML-130, 5 μm	107 ± 11	100 ± 15	108 ± 10
BML-130, 50 μm	160 ± 21[a]	100 ± 15	106 ± 29
IV-231B, 5 μm	106 ± 3	116 ± 20	90 ± 8
IV-231B, 50 μm	113 ± 8	112 ± 3	71 ± 18[a]

[a]Notable differences.

Assay of the two kinds of ceramidase (Table 6) showed that there was no effect of either the aliphatic or aromatic inhibitors at the 5 μM level, at which point cell growth is completely stopped in the case of the pyrrolidino compounds. At the 50 μM level, however, the acid enzyme was stimulated markedly by the aromatic inhibitors, particularly the two stereoisomeric forms of the pyrrolidino compound.

Sphingomyelin synthase was unaffected by PDMP or the aliphatic inhibitor but BML-129 and -130 produced appreciable inhibition at 50 μM (54% and 61%, respectively) (Table 7).

TABLE 7

Effect of Inhibitors on Acid and Neutral Sphingomyelinases and Sphingomyelin Synthase

Inhibitor Tested	Enzyme Activity (% of control) Sphingomyelinase pH 4.5	Sphingomyelinase pH 7.1	Sphingo-myelinase Synthase[a]
D-threo-PDMP, 5 μM	102 ± 3	121 ± 13	
D-threo-PDMP, 50 μM	100 ± 3	108 ± 8	
BML-129, 5 μM	108 ± 4	105 ± 11	84 ± 27
BML-129, 50 μM	97 ± 3	142 ± 11[b]	46 ± 11[b]
BML-130, 5 μM	109 ± 1	110 ± 7	87 ± 14
BML-130, 50 μM	114 ± 2	152 ± 14	39 ± 18[b]
IV-231B, 5 μM	101 ± 7	131 ± 3[b]	
IV-231B, 50 μM	112 ± 11	120 ± 3[b]	

[a]Data for PDMP and IV-231B are not shown here as they were tested in other experiments; no effect was seen.
[b]Notable differences.

Neutral sphingomyelinase (SMase) was distinctly stimulated by the aliphatic inhibitor, IV-231B, even at 5 μM (Table 7). From this one would expect that the inhibitor would produce accumulation of ceramide, yet it did not. The two pyrrolidino compounds produced appreciable stimulation at the 50 μM level. No significant effects were obtained with acid SMase.

Discussion

The present invention shows that the nature and size of the tertiary amine on ceramide-like compounds exerts a strong influence on GlcCer synthase inhibition, a 5-membered ring being most active. It also shows that the phenyl ring used previously to simulate the trans-alkenyl chain corresponding to that of sphingosine could, with benefit, be replaced with the natural alkenyl chain.

Findings with the most active GlcCer synthase inhibitors in growth tests compare favorably with evaluations of some clinically useful chemotherapeutic agents on three of the tumor cell lines in the same Drug Evaluation Core Laboratory. The IC_{50} values were 0.2 to 6 μM for cisplatin, 0.02 to 44 μM for carboplatin, 0.03 to 0.2 μM for methotrexate, 0.07 to 0.2 μM for fluorouracil, and 0.1 to 1 μM for etoposide. Unlike these agents, the compounds of the present invention yielded rather similar effects with all the cell types, including MDCK cells, and thus have wider potential chemotherapeutic utility. This uniformity of action is consistent with the idea that GSLs play a wide and consistent role in cell growth and differentiation.

An important observation from the MDCK cell study is that strong inhibition of cell growth and DNA synthesis occurred only at the same concentrations of aromatic inhibitor that produced marked ceramide accumulation. This observation supports the assertion that ceramide inhibits growth and enhances differentiation or cell death (Bielawska, A. et al., "Modulation of Cell Growth and Differentiation by Ceramide," *FEBS Letters,* 307:211–214 (1992)). It also agrees with previous work with octanoyl sphingosine, a short chain ceramide that produced greatly elevated levels of natural ceramide and slowed growth (Abe, A. et al., "Metabolic Effects of Short-Chain Ceramide and Glucosylceramide on Sphingolipids and Protein Kinase C," *Eur. J. Biochem.,* 210:765–773 (1992)). It is also in agreement with a finding that some synthetic, nonionic ceramide-like compounds did not inhibit GlcCer synthase even though they behave like ceramide in blocking growth (Bielawska, A. et al., "Ceramide-Mediated Biology. Determination of Structural and Stereospecific Requirements Through the Use of N-Acyl-Phenylaminoalcohol Analogs," *J. Biol. Chem,.* 267:18493–18497 (1992)). Compounds tested included 20 μM D-erythro-N-myristoyl-2-amino-1-phenyl-1-propanol, its L-enantiomer, the four stereoisomers of N-acetylsphinganine, and N-acetylsphingosine. Furthermore, the lack of growth inhibition and ceramide accumulation in cells treated with the aliphatic inhibitor IV-231B is also consistent with the correlation between ceramide level and growth rate.

The accumulation of ceramide that occurred at higher levels of GlcCer synthase inhibitors could be attributed not only to blockage of ceramide utilization, but also to blockage of SM synthesis or ceramide hydrolase. This possibility is especially relevant to the R,S-, S,R-, and S,S-isomers, which seem to exert effects on sphingolipids without strongly inhibiting GlcCer synthesis. The tests with both the DL-erythro-pyrrolidino inhibitor (BML-129) and the DL-threo-pyrrolidino inhibitor (BML-130), at a level producing strong growth inhibition, showed that neither material at a low concentration inhibited the enzymes tested in vitro (Tables 6 and 7) but they did cause growth inhibition as well as accumulation of ceramide. PDMP, at relatively high concentrations (50 μM), was found to inhibit SM synthase in growing CHO cells (Rosenwald, A. G. et al., "Effects of a Sphingolipid Synthesis Inhibitor on Membrane Transport Through the Secretory Pathway," *Biochemistry,* 31:3581–3590 (1992)). In the test with MDCK homogenates, it did not inhibit this synthase, in agreement with the finding that labeled palmitate incorporation into SM was stimulated by PDMP (Shayman, J. A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.,* 266:22968–22974 (1991)).

Retinoic acid is a growth inhibitor of interest in cancer chemotherapy and a possible adjunct in the use of the inhibitors of the present invention. It has been found to elevate ceramide and DAG levels (Kalen, A. et al., "Elevated Ceramide Levels in GH4C1 Cells Treated with Retinoic Acid," *Biochim. Biophys. Acta,* 1125:90–96 (1992)) and possibly lower lecithin content (Tang, W. et al., "Phorbol Ester Inhibits 13-Cis-Retinoic Acid-induced Hydrolysis of Phosphatidylinositol 4,5-Bisphosphate in Cultured Murine Keratinocytes: a Possible Negative Feedback Via Protein Kinase C-Activation," *Cell Bioch. Funct.,* 9:183–191 (1991)).

D-threo-PDMP was as found to be rather active in delaying tumor cell growth or in producing complete cures in mice (Inokuchi, J. et al., "Antitumor Activity in Mice of an Inhibitor of Glycosphingolipid Biosynthesis," *Cancer Lett,.* 38:23–30 (1987)) but high doses were needed. From the data in FIG. **1**, the inhibitors of the present invention are approximately 30 times as active, so the dosage levels are typical of clinically useful drugs. The need to use high doses with PDMP was attributed to rapid inactivation by cytochrome P450 (Shukla, A. et al., "Metabolism of D-[^{3}H]PDMP, an Inhibitor of Glucosylceramide Synthesis, and the Synergistic Action of an Inhibitor of Microsomal Monooxygenase," *J. Lipid Res.,* 32:713–722 (1991)). Cytochrome P450 can be readily blocked by various nontoxic drugs such as cimetidine, therefore high levels of the compounds of the present invention can be maintained.

SPECIFIC EXAMPLE 2

A series of inhibitors based on substitutions in the phenyl ring of P4 were synthesized and studied. It was found that the potency of the inhibitors in blocking GlcCer synthase was mainly dependent upon hydrophobic and electronic properties of the substituent. Surprisingly, a linear relationship was found between log [IC_{50}] and hydrophobic parameter (π)+electronic parameter (δ). This correlation suggested that electron donating and hydrophilic characters of the substituent enhance the potency as an inhibitor. This observation resulted in the synthesis of novel compounds that are more active in blocking glucosylceramide formation. Two compounds, dioxy D-t-P4 compounds, D-t-3',4'-ethylenedioxy-P4 and D-t-4'-hydroxy-P4, were observed to be significantly more potent than other tested inhibitors. In particular, at 11.3 nM D-t-3',4'-ethylenedioxy-P4, 80% of glucosylceramide in MDCK cell was depleted without any ceramide accumulation and cell growth inhibition. The potency of D-t-3',4'-ethylenedioxy-P4 appears to be not only regulated by hydrophobic and electronic properties but also by stearic properties of the substituents on the phenyl group.

Materials and Methods

Materials. The acetophenones and amines were from Aldrich Chemical Co., St. Louis, Mo., Lancaster Synthesis Inc., Windham, N.H. and Maybridge Chemical Co., Cornwall, UK. Silica gel for column chromatography (70–230 mesh ASTM) and Silica gel thin layer chromatography plates were purchased from Merck Co. The reagents and their sources were: non-hydroxy fatty acid ceramide from bovine brain and delipidated bovine serum albumin (BSA) from Sigma; dioleoyphosphatidylcholine from Avanti; DL-dithiothreitol from Calbiochem; 1-[^{3}H]-glucose uridine diphosphate from NEN. Octanoylsphingosine, glucosylceramide and sodium sulfatide were prepared as previously described. Abe, A. et al., *Eur. J. Biochemistry,* 210:765–773 (1992).

General synthesis of inhibitors. The aromatic inhibitors were synthesized by the Mannich reaction from 2-N-

acylaminoacetophenone, paraformaldehyde, and pyrrolidine, and then the reduction from sodium borohydride as described before. Inokuchi, J. et al., *J. Lipid. Res.,* 28:565–571 (1987); Abe, A. et al., *J. Lipid. Res.,* 36:611–621 (1995). The reaction produces a mixture of four isomers, due to the presence of two asymmetric centers. For these syntheses in which phenyl-substituted starting materials were used, the chloro, methoxy, methylenedioxy, methyl groups in the acetophenone structure were brominated and converted to the primary amine. Bromation of the methoxyacetophenone, dimethyoxyacetophenone, 3',4'-(methylenedioxy)acetophenone were performed in chloroform at room temperature and recrystallized from ethyl acetate and hexane.

Synthesis of 1-(4'-hydroxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol. The synthesis of 1-(4'-hydroxy) phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol is described in detail in FIG. **8**. This synthesis differs from those of the other compounds because of the need for the placement of a protecting group on the free hydroxyl (step 1) and its subsequent removal (step 7). All other syntheses employ a similar synthetic scheme (steps 2 to 6).

4'-Benzyloxyacetophenone formation (step 1) 4'-Hydroxyacetophenone (13.62 g, 100 mmol), benzylbromide (17.1 g, 100 mmol), and cesium carbonate (35.83 g, 100 mmol) were added to tetrahydrofuran at room temperature and stirred overnight. The product was concentrated to dryness and recrystallized from ether and hexane to yield 15 g of 4'-benzyloxyacetophenone which appeared as a white powder. An R_f of 0.42 was observed when resolved by thin layer chromatography using methylene chloride. ^{1}H nmr (δ, ppm, $CDCl_3$), 7.94 (2H, δ, 8.8 Hz, O—Ar—C(O)), 7.42 (5H, m, Ar'CH_2O—), 7.01 (2H, δ, 8.8 Hz, O—Ar—C(O)), 5.14 (2H, s, Ar'CH_2O—), 2.56 (3H, S, CH_3).

Bromination of 4'-benzyloxyacetophenone (step 2) Bromine (80 mmol) was added dropwise over 5 min to a stirred solution of 4'-benzyloxyacetophenone (70 mmol) in 40 ml chloroform. This mixture was stirred for an additional 5 min and quenched with saturated sodium bicarbonate in water until the pH reached 7. The organic layers were combined, dried over $MgSO_4$, and concentrated to dryness. The crude mixture was purified over a silica gel column and eluted with methylene chloride to yield 2-bromo-4'-benyloxyacetophenone. An R_f of 0.62 was observed when resolved by thin layer chromatography using methylene chloride. ^{1}H nmr (δ, ppm, $CDCl_3$), 7.97 (2H, δ, 9.2 Hz, O—Ar—C(O)), 7.43 (5H, m, Ar'CH_2O—), 7.04 (2H, δ, 9.0 Hz, O—Ar—C(O)), 5.15 (2H, s, Ar'CH_2O—), 4.40 (2H, s, CH_2 Br).

2-Amino-4'-benzyloxyacetophenone HCl formation (step 3) Hexamethylenetetramine (methenamine, 3.8 g, 23 mmol) was added to a stirred solution of 2-bromine4'-benyloxyacetophenone (6.8 g, 23 mmol) in 100 ml chloroform. After 4 h the crystalline adduct was filtered and washed with chloroform. The product was dried and heated with 150 ml methanol and 8 ml of concentrated HCl in an oil bath at 85° C. for 3 h. Upon cooling the precipitated hydrochloride salt (2.5 g) was removed by filtration. The filtrate was left at −20° C. overnight and additional product (2.1 g) was isolated. The yield was 4.6 g (82.6%). $[M^+H]^+$: 242 for $C_{15}H_{16}NO_2$. ^{1}H nmr (δ, ppm, $CDCl_3$), 8.38 (2H, bs, NH_2), 7.97 (2H, δ, 8.8 Hz, O—Ar—(O)), 7.41 (5H, m, Ar'CH_2O—), 7.15 (2H, δ, 8.6 Hz, OArC(O)), 5.23 (2H, s, Ar'CH_2O), 4.49 (2H, s, CH_2 NH_2).

2-Palmitoylamino-4'-benyloxyacetophenone formation (step 4) Sodium acetate (50% in water, 29 ml) was added in three portions to a stirred solution of 2-amino-4'-benzyloxyacetophenone HCl (4.6 g, 17 mmol) and tetrahydrofuran (200 ml). Palmitoyl chloride (19 mmol) in tetrahydrofuran (25 ml) was added dropwise over 20 min yielding a dark brown solution. The mixture was stirred overnight at room temperature. The aqueous fraction was removed by use of a separatory funnel and chloroform/methanol (2/1, 150 ml) was added to the organic layer which was then washed with water (50 ml). The yellow aqueous layer was extracted once with chloroform (50 ml). The organic solutions were then pooled and rotoevaporated until near dryness. The residue was redissolved in chloroform (100 ml) and crystallized by the addition of hexane (400 ml). The flask was cooled to 4° C. for 2 h. The crystals were filtered and washed with cold hexane and dried in a fume hood overnight. The product yield was 3.79 g (8 mmol). An R_f of 0.21 was observed when resolved by thin layer chromatography using methylene chloride. $[M^+H]^+$: 479 for $C_{31}H_{45}NO_3$. ^{1}H nmr (δ, ppm, $CDCl_3$), 7.96 (2H, δ, 8.8 Hz, O—Ar—C(O)), 7.40 (5H, m, Ar'CH_2O—), 7.03 (2H, δ, 8.8 Hz, O—Ar—C(O)), 6.57 (1H, bs, NH_2), 5.14 (2H, s, Ar'CH_2O—), 4.71 (2H, s, C(O)CH_2NHC(O)), 2.29 (2H, t, 7.4 Hz, C(O)$CH_2(CH_2)_{13}CH_3$), 1.67 (2H, m, C(O)CH_2 $(CH_2)_{13}CH_3$), 0.87 (3H, t, 6.7 Hz, C(O)$CH_2(CH_2)_{13}CH_3$).

1-(4'-Benzyloxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol formation (steps 5 and 6) 2-Palmitoylamino-4'-benzyloxyacetophenone (3.79 g, 8.0 mmol), paraformaldehyde (0.25 g, 2.7 mmol), pyrrolidine (0.96 ml, 11.4 mmol) and ethanol (70 ml) were stirred under nitrogen. Concentrated HCl (0.26 ml) was added through the condensor and the mixture was heated to reflux for 16 h. The resultant brown solution was cooled on ice and then sodium borohydride (1.3 g, 34 mmol) was added in three portions. The mixture was stirred at room temperature overnight, and the product was dried in a solvent evaporator. The residue was redissolved in dichloromethane (130 ml) and hydrolyzed with 3N HCl (pH~4). The aqueous layer was extracted twice with dichloromethane (50 ml). The organic layers were pooled and washed twice with water (30 ml), twice with saturated sodium chloride (30 ml), and dried over anhydrous magnesium sulfate. The dichloromethane solution was rotoevaporated to a semisolid and purified by use of a silica rotor using a solvent consisting of 10% methanol in dichloromethane. This yielded a mixture of DL-threo- and DL-erythro enantiomers (2.53 g, 4.2 mmol). An R_f of 0.43 for the erythro diastereomers and 0.36 for the threo diastereomers was observed when resolved by thin layer chromatography using methanol:methylene chloride (1:9). $[M^+H]^+$: 565 for $C_{36}H_{56}N_2O_3$.

1-(4'-Hydroxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol formation (step 7): A suspension of 20% Pd/C (40 mg) in acetic acid (15 ml) was stirred at room temperature under a hydrogen balloon for 15 min. 1-(4'-Benzyloxy) phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (420 mg, 0.74 mmol) was added and the solution was stirred overnight. The suspension was filtered through a glass frit, and the filter was rinsed with acetic acid:methylene chloride (1:1, 5 ml). The filtrate was concentrated in vacuo and crystallized to yield a pale yellow semisolid (190 mg, 0.4 mmol). An R_f of 0.21 was observed when resolved by thin layer chromatography using methanol:methylene chloride (1:9). $[M^+H]^+$: 475 for $C_{29}H_{50}N_2O_3$. ^{1}H nmr (δ, ppm, $CDCl_3$), 7.13 (4H, m, ArCHOH—), 7.14 (1H, δ6.9 Hz, —NH—), 5.03 (1H, δ, 3.3 Hz, CHOH—), 4.43 (1 H, m, c-$(CH_2CH_2)_2NCH_2$CH), 3.76 (2H, m, c-$(CH_2CH_2)_2$N—), 3.51 (1H, m, c-$(CH_2CH_2)_2NCH_2$—), 3.29 (1 H, m, c-$(CH_2CH_2)_2NCH_2$—), 2.97 (3H, m, c-$(CH_2CH_2)_2$N— and

ArC(OH)H—), 2.08 (6H, m, —$C(O)CH_2(CH_2)_{13}CH_3$ and c-$(CH_2CH_2)_2N$—, 1.40 (2H, m, $C(O)CH_2CH_2(CH_2)_{12}CH_3$), 1.25 (2H, m, —$C(O)CH_2CH_2(CH_2)_{12}CH_3$), 0.87 (3H, t, 6.7 Hz, $C(O)CH_2(CH_2)_{13}CH_3$).

Synthesis of D-threo-1-(3',4'-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol.

2-Amino-3',4'-(ethylenedioxy)acetophenone HCl: Hexamethylenetetramine (methenamine, 5.4 g, 0.039 mol) was added to a stirred solution of phenacylbromide (10.0 g, 0.039 mol) in 200 ml chloroform. After 2 h, the crystalline adduct was filtered and washed with chloroform. The product was then dried and heated with methanol (200 ml) and concentrated HCl (14 ml) in an oil bath at 85° C. for 2 h. On cooling, the precipitated ammonium chloride was removed by filtration and the filtrate was left in a freezer overnight. After filtration the crystallized phenacylamine HCl was washed with cold isopropanol and then with ether. The yield of this product was ~7.1 g (81%).

2-Palmitoylamino-3',4'-(ethylenedioxy)acetophenone: Aminoacetophenone HCl (7.1 g, 31 mmol) and tetrahydrofuran (300 ml) were placed in a 1 liter three-neck round bottom flask with a large stir bar. Sodium acetate (50% in water, 31 ml) was added in three portions to this suspension. Palmitoyl chloride (31 ml, 10% excess, 0.036 mol) in tetrahydrofuran (25 ml) was then added dropwise over 20 min to yield a dark brown solution. This mixture was then stirred for an additional 2 h at room temperature. The resultant mixture was poured into a separatory funnel to remove the aqueous solution. Chloroform/methanol (2/1, 150 ml) was then added to the organic layer and washed with water (50 ml). The yellow aqueous layer was extracted once with chloroform (50 ml). The organic solutions were pooled and rotoevaportated until almost dry. The residue was redissolved in chloroform (100 ml) and crystallized by the addition of hexane (400 ml). The flask was then cooled to 4° C. for 2 h. The crystals were filtered and washed with cold hexane until they were almost white and then dried in a fume hood overnight. The yield of the product was 27 mmol (11.6 g).

D-threo-1-(3 ',4'-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-prop anol: Almitoylaminoacetophenone (11.6 g, 0.027 mol), paraformaldehyde (0.81 g, 0.009 mol), pyrrolidine (3.6 ml, 0.042 mol) and ethanol (250 ml) were added to a 500 ml round flask under nitrogen flow. Concentrated HCl (0.8 ml) was added to this mixture through the reflux condenser and the mixture was refluxed for 16 h. The brown solution was cooled in an ice-bath. Sodium borohydride (2.28 g, 0.06 mol) was added in three portions. This mixture was stirred at room temperature for 3 h and then rotoevaporated. The residue was dissolved in 130 ml of dichloromethane and the borate complex hydrolyzed with HCl (3N) until the pH was approximately 4. The aqueous layer was extracted twice with 50 ml dichloromethane. The organic layers were pooled and washed twice with H_2O (30 ml), saturated NaCl (30 ml) and dried over anhydrous $MgSO_4$. The dichloromethane solution was rotoevaporated to a viscous oil which was purified by use of a Chromatotron with a solvent consisting of 10% methanol in dichloromethane to obtain a mixture of DL-threo and erythro enantiomers (2.24 g, 0.004 mol).

Resolution of inhibitor enantiomers. High performance liquid chromatography (HPLC) resolution of the enantiomers of DL-threo and DL-erythro are performed using a preparative HPLC column (Chirex 3014: [(S)-val-(R)-1-(a-naphtyl)ethylamine, 20×250 mm: Phenomenex], eluted with hexane-1,2-dichloroethane-ethanol-trifluroacetic acid 64:30:5.74:0.26, at a flow rate of 8 ml/min. The column eluent was monitored at 254 nm in both the preparative and analytical modes. Isolated products were reinjected until pure by analytical HPLC analysis, determined using an analytical Chirex 3014 column (4.6×250 mm) and the above solvent mixture at flow rate of 1 ml/min.

Glycosylceramide synthase activity. The enzyme activity was measured by the method previously described in Skukla, G. et al., *Biochim. Biophys. Acta,* 1083:101–108 (1991). MDCK cell homogenate (120 μg of protein) was incubated with uridinediphosphate [^{3}H]glucose (100,000 cpm) and liposomes consisting of 85 μg octanoylsphingosine, 570 μg dioleoyphosphatidylcholine and 100 μg sodium sulfatide in 200 μl of reaction mixture and kept for 1 h at 37° C. P4 and P4 derivatives dissolved in dimethyl sulfoxide were dispersed into the reaction mixture after adding liposomes. The final concentration of dimethyl sulfoxide was kept 1% under which the enzyme activity was not at all inhibited.

Cell culture and lipid extraction. One half million of MDCK cells were seeded into 10 cm style dish containing 8 ml serum free DMEM supplemented medium. Shayman, J. A. et al., *J. Biol. Chem.,* 265:12135–12138 (1990). After 24 h the medium was replaced with 8 ml of the medium containing 0, 11.8, 118 or 1180 nM D-t-P4, D-t-3',4'-ethylenedioxy-P4 or D-4'-hydroxy)-P4. The GlcCer synthase inhibitors were added into the medium as a one to one complex with delipidated BSA. Abe, A. et al., *J. Lipid. Res.,* 36:611–621 (1995); Abe, A. et al., *Biochim. Biophys. Acta,* 1299:331–341 (1996). The cells were incubated for 24 h or 48 h with the inhibitors. After the incubation, the cells were washed twice with 8 ml of cold PBS and fixed with 2 ml of cold methanol. The fixed cells were scraped and transferred to a glass tube. Another one ml of methanol was used to recover the remaining cells in the dish.

Three ml of chloroform was added to the tube and briefly sonicated using a water bath type sonicator. After centrifugation at 800 g for 5 min, the supernatant was transferred into another glass tube. The residues were reextracted with chloroform/methanol (1/1). After the centrifugation, the resultant supernatant was combined with the first one. The residues were air-dried and kept for protein analysis. Adding 0.9% NaCl to the supematant combined, the ratio of chloroform/methanol/aqueous was adjusted to 1/1/1. After centrifugation 800 g for 5 min, the upper layer was discarded. Methanol/water (1/1) with the same amount of volume of the lower layer was used to wash. The resultant lower layer was transferred into a small glass tube and dried down under a stream of nitrogen gas. A part of the lipid was used for lipid phosphate determination. Ames, B. N., *Methods Enzymol.,* 8:115–118 (1966). The remainder was analyzed using HPTLC (Merck).

Results

Synthesis of P4 and P4 derivatives. The preparation of P4 derivatives utilized the Mannich reaction from 2-N-acylaminoacetophenone, paraformaldehyde, and pyrrolidine, and then the reduction of DL-pyrrodino ketone from sodium borohydride. In most cases, no isolation of DL-pyrrodino ketones were performed to maintain solubility. The overall yields of the DL-threo and DL-erythro syntheses were ~10–30%. These derivatives were purified by the either silica gel column or rotors with solvent 5–12% methanol in dichloromethane to optimize the separation from the chiral column. To obtain the best separation, each injection contains no more than 150 mg, and fractions were

pooled to obtain sufficient quantity of isomer of D-threo for further biological characterization.

Resolution of PDMP homologues by chiral chromatography. The structures of the parent compound, D-threo-P4 and the phenyl-substituted homologues including the new dioxy-substituted and 4'-hydroxy-P4 homologues are shown in FIG. **9**. Initially the effect of each P4 isomer separated by chiral chromatography on GlcCer synthase activity was determined (FIG. **10**). Four peaks were observed for the chiral separation of P4. Peaks 1 and 2 represented the erythro diastereomers and 3 and 4 represented the threo diastereomers as determined by a sequential separation of the P4 mixture by reverse phase chromatography followed by the chiral separation. The enzyme activity was specifically inhibited by the fourth peak, the D-threo isomer (FIG. **4**A). This specificity for the D-threo enantiomer was consistent with the previous results observed in PDMP and PDMP homologues (2–4). The IC_{50} of D-threo-P4 was 0.5 mM for GlcCer synthase activity measured in the MDCK cell homogenates.

Effects of P4 and P4 Derivatives with a Single Substituent of Phenyl Group on GlcCer Synthase Activity. The effect of each P4 isomer on GlcCer synthase activity was analyzed. The reaction was carried out in the absence or presence of 0.1, 1.0 or 10 μM P4 (FIG. **4**A) or p-methoxy-P4 (FIG. **4**B). As shown in FIG. **4**A, the enzyme activity was specifically inhibited by D-threo isomer. In FIG. **4**A, the symbols are denoted as follows: D-threo (○), D-erythro (□), L-threo and (●), L-erythro (Δ). This specificity is consistent with previous results observed in PDMP and PDMP homologs. Inokuchi, J. et al., *J. Lipid. Res.* 28:565–571 (1987); Abe, A et al., *J. Lipid. Res.* 36:611–621 (1995). The IC_{50} of D-t-P4 was 500 nM.

As set forth herein, the addition of a p-methoxy group to DL-t-P4 was found to enhance the effect of the inhibitor on the enzyme activity. Abe, A. et al., *J. Lipid. Res.,* 36:611–621 (1995). As shown in FIG. **4**B, it was confirmed that the enzyme activity was potently inhibited by D-threo-p-methoxy-P4 whose IC_{50} was 200 nM. In FIG. **4**B, □ denotes a mixture of D-erythro and L-threo isomers contaminated with a small amount of the D-threo isomer. Chiral chromatography of the four p-methoxy-P4 enantiomers failed to completely resolve to baseline each enantiomer (FIG. **10**). A slight inhibition of the enzyme activity by p-methyoxy-P4 in a combined D-erythro and L-threo mixture (peaks 2 and 3, FIG. **10**) was observed; this was due to contamination of the D-threo isomer (peak 4, FIG. **10**) into these fractions.

A series of D-t-P4 derivatives containing a single substituent on the phenyl group were investigated. As shown in Table 8, the potency of the derivatives as inhibitors were inferior to that of D-t-P4 or p-methoxy-D-t-P4. In many drugs, the influence of an aromatic substituent on the biological activity has been known and predicted. Hogberg, T. et al., Theoretical and experimental methods in drug design applied on antipsychotic dopamine antagonists. Larsen, P. K., and Bundgaard, H., "Textbook of Drug Design and Development," pp. 55–91 (1991). Generally IC_{50} is described as the following equation:

$$\log(1/IC_{50})=a(\text{hydrophobic parameter } (\pi))+b(\text{electronic parameter } (\sigma))+c(\text{stearic parameter})+d(\text{other descriptor})+e$$

where a, b, c, d and e are the regression coefficients. Hogberg, T. et al., Theoretical and experimental methods in drug design applied on antipsychotic dopamine antagonists. Larsen, P. K., and Bundgaard, H., "Textbook of Drug Design and Development," pp. 55–91 (1991).

The hydrophobic effect, π, is described by the equation π-log P_X −log P_H where P_X is the partition coefficient of the substituted derivative and P_H is that of the parent compound, measured as the distribution between octanol and water.

The electronic substituent parameter, σ, was originally developed by Hammett (Hammett, L. P., In Physical Organic Chemistry, McGraw-Hill, New York (1940)) and is expressed as $\sigma=\log K_X - \log K_H$, where K_X and K_H are the ionization constants for a para or meta substituted derivative and benzoic acid respectively. Positive σ values represent electron withdrawing properties and negative σ values represent electron donating properties.

The potency of D-threo-P4 and P4 derivatives as an inhibitor is mainly dependent upon two factors, hydrophobic and electronic properties, of a substituent of phenyl group (Table 8). Surprisingly, a linear relationship was observed between log (IC_{50}) and $\pi+\sigma$ (FIG. **5**). These findings suggest that the more negative the value of $\pi+\sigma$, the more potent is D-threo-P4 derivatives made as GlcCer synthase inhibitor.

The data in Table 8 indicate that the potency of D-t-P4 and P4 derivatives as an inhibitor is mainly dependent upon two properties, hydrophobic and electronic properties, of a substituent of the phenyl group. Surprisingly, a linear relationship was observed between log(IC_{50}) and $\pi+\sigma$ (FIG. **5**). These findings suggest that the more negative the value of $\pi+\sigma$, the more potent the D-t-P4 derivative as a GlcCer synthase inhibitor.

TABLE 8

D-threo-P4 derivative	$\sigma + \pi$*	IC_{50} (μM)**
p-methoxy	−0.29	0.2
P-4	0.00	0.5
m-methoxy-P4	0.10	0.6
p-methyl-P4	0.39	2.3
p-chloro-P4	0.94	7.2

*These values were estimated from the Table in Hogberg, T. et al., Theoretical and experimental methods in drug design applied on antipsychotic dopamine antagonists. Larsen, P. K., and Bundgaard, H., "Textbook of Drug Design and Development," pp. 55–91 (1991), for methoxy, σ_m 0.12, σ_p = −0.27, π = −0.02; hydro, σ = 0, π = 0; methyl, σ_p = −0.17, π = 0.56; chloro, σ_p = 0.23, π = 0.71.
**These values were derived from FIGS. 4A and 4B. For other compounds the same analytical approach as shown in FIGS. 4A and 4B was carried out to obtain the IC_{50}.

The p-hydroxy-substituted homologue was a significantly better GlcCer synthase inhibitor. The strong association between $\pi+\sigma$ and GlcCer synthase inhibition suggested that a still more potent inhibitor could be produced by increasing the electron donating and decreasing the lipophilic properties of the phenyl group substituent. A predictably negative $\pi+\sigma$ value would be observed for the p-hydroxy homologue. This compound was synthesized and the D-threo enantiomer isolated by chiral chromatography. An IC_{50} of 90 nM for GlcCer synthase inhibition was observed (FIG. **11**), suggesting that the p-hydroxy homologue was twice as active as the p-methoxy compound. Moreover, the linear relationship between the log (IC_{50}) and $\pi+\sigma$ was preserved (open circle, FIG. **4**).

Effects of 3',4'-dioxy-D-threo-P4 Derivatives on GlcCer Synthase Activity. The result in FIG. **5** suggested that an electron donating and hydrophilic substituent of phenyl group makes the GlcCer synthase inhibitor potent. To attain further improvement of the inhibitor, another series of P4 derivatives with methylenedioxy, ethylenedioxy and trimethyidioxy substitutions on the phenyl group were designed (FIG. **9**).

As shown in FIG. **6**, the enzyme activity was markedly inhibited by D-t-3',4'-ethylenedioxy-P4 whose IC_{50} was 100

nM. In FIG. **6**, □ denotes D-t-3',4'-methylenedioxy-P4, ○ denotes D-t-3',4'-ethylenedioxy-P4, Δ denotes D-t-3',4'-trimethylenedioxy-P4 and ● denotes D-t-3',4'-dimethyoxy-P4. One the other hand, the IC_{50} s for D-t-3',4'-methylenedioxy-P4 and D-t-3',4'-trimethylenedioxy-P4 were about 500 and 600 nM, respectively. These results suggest that the potency of D-t-3',4'-ethylenedioxy-P4 is not only regulated by hydrophobic and electronic properties but also by other factors, most likely stearic properties, induced from the dioxy ring on the phenyl group.

Interestingly, D-t-3',4'-dimethoxy-P4 was inferior to these dioxy derivatives, even to D-t-P4 or m- or D-t-p-methoxy-P4, as an inhibitor (FIG. **6**). As the parameters, σ_m, σ_p and π, for methoxy substituent are 0.12, −0.27 and −0.02, respectively (Hogberg, T. et al., Theoretical and experimental methods in drug design applied on antipsychotic dopamine antagonists. Larsen, P. K., and Bundgaard, H., "Textbook of Drug Design and Development," pp. 55–91 (1991)), the value of $\pi+\sigma$ of D-t-dimethoxy P4 is presumed to be negative. Therefore, the dimethoxy-P4 is thought to deviate quite far from the correlation as observed in FIG. **5**. There may be a repulsion between two methoxy groups in the dimethoxy-P4 molecule that induces a stearic effect that was negligible in mono substituent D-t-P4 derivatives studied in FIG. **5**. GlcCer synthase is thought to possess a domain that interacts with D-t-PDMP and PDMP homologs and that modulates the enzyme activity. Inokuchi, J. et al., *J. Lipid. Res.,* 28:565–571 (1987); Abe, A. et al., *Biochim. Biophys. Acta,* 1299:331–341 (1996). The stearic effect generated by an additional methoxy group may affect the interaction between the enzyme and the inhibitor. As a result, the potency as an inhibitor is markedly changed.

Distinguishing Between Inhibition of GlcCer Synthase and 1-O-acylceramide Synthase Inhibition. Prior studies on PDMP and related homologues revealed that both the threo and erythro diastereomers were capable of increasing cell ceramide and inhibiting cell growth in spite of the observation that only the D-threo enantiomers blocked GlcCer synthase. An alternative pathway for ceramide metabolism was subsequently identified, the acylation of ceramide at the 1-hydroxyl position, which was blocked by both threo and erythro diastereomers of PDMP. The specificities of D-threo-P4, D-threo-3',4'-ethylenedioxy-P4, and D-threo-(4'-hydroxy)-P4 for GlcCer synthase were studied by assaying the transacylase. Although there was an ca. 100 fold difference in activity between D-threo-3',4'-ethylenedioxy-P4, D-threo-(4'-hydroxy)-P4, and D-threo-P4 (IC_{50} 0.1 mM versus 10 mM) in inhibiting GlcCer synthase, the D-threo enantiomers of all three compounds demonstrated comparable activity in blocking 1-O-acylceramide synthase (FIG. **12**).

In order to determine whether inhibition of 1-O-acylceramide synthase was the basis for inhibitor mediated ceramide accumulation, the ceramide and diradylglycerol levels of MDCK cells treated D-threo-P4, D-threo-3',4'-ethylenedioxy-P4, and D-threo-(4'-hydroxy)-P4 were measured (Table 9). MDCK cells (5×10.sup.5) were seeded into a 10 cm dish and incubated for 24 h. Following the incubation, the cells were treated for 24 or 48 h with or without P4 or the phenyl substitute homologues. Both ceramide and diradylglycerol contents were determined by the method of Preis, J. et al., *J. Biol. Chem.,* 261:8597–8600 (1986). GlcCer content was measured densitometrically by a video camera and use of NIH image 1.49. Significant increases in both ceramide and diradylglycerol occurred only in cells treated with inhibitor concentrations in excess of 1 mM. This was approximately 30-fold lower than the

concentration required for inhibition of the 1-O-acylceramide synthase assayed in the cellular homogenates. This disparity in concentration effects most likely reflects the ability of the more potent homologues to accumulate within intact cells. Abe, A. et al., *Biochim. Biophys. Acta,* 1299:331–341 (1996).

TABLE 9

GlcCer, ceramide and diradylglycerol content of MDCK cells treated with D-threo-P4, D-threo-3',4'-ethylenedioxy-P4, and D-threo-(4'-hydroxy)-P4

	Condition	Ceramide (pmol/nmol phospholipids)	Diradylglycerol (pmol/nmol phospholipids)
Control	24 h	4.53 ± 0.12	24.2 ± 2.36
	48 h	6.68 ± 0.49	32.3 ± 3.11
D-threo-P4	11.3 nM		
	24 h	5.33 ± 0.41*	24.1 ± 1.66
	48 h	5.68 ± 0.27*	29.6 ± 0.73
	113 nM		
	24 h	4.64 ± 0.38	26.6 ± 1.56
	48 h	7.08 ± 0.29	33.0 ± 2.63
	1130 nM		
	24 h	5.10 ± 0.35	27.1 ± 0.67
	48 h	9.74 ± 0.53	38.8 ± 1.11
D-threo-4'-hydroxy-P4	11.3 nM		
	24 h	4.29 ± 0.71	30.9 ± 2.01*
	48 h	6.70 ± 0.29	38.4 ± 1.44*
	113 nM		
	24 h	5.09 ± 0.95	31.5 ± 3.84*
	48 h	7.47 ± 0.29	41.5 ± 0.66*
	1130 nM		
	24 h	7.38 ± 0.13	38.5 ± 3.84*
	48 h	13.4 ± 1.03*	47.2 ± 2.51*
D-threo-3',4'-ethylenedioxy-P4	11.3 nM		
	24 h	5.24	22.0
		5.04	24.7
	113 nM		
	24 h	5.21	32.5
		5.21	41.6
	1130 nM		
	24 h	9.64	32.5
		13.0	41.6

*Denotes $p < 0.05$ by the Student t test. For the D-threo-(ethylenedioxy)-P4 only two determinations were made.

Effects of D-threo-P4, D-threo4'-hydroxy-P4 and D-threo-3,4'-ethylenedioxy-P4 on GlcCer Synthesis and Cell Growth. To confirm the cellular specificity of D-threo-3',4'-ethylenedioxy-P4 and D-threo-(4'-hydroxy)-P4 as compared to D-threo-P4, MDCK cells were treated with different concentrations of the inhibitors. The total protein amount in each sample was determined by the BCA method. In GlcCer analysis, lipid samples and standard lipids were applied to the same HPTLC plate pre-treated with borate and developed in a solvent consisting of C/M/W (63/24/4). The level of GlcCer was estimated from a standard curve obtained using a computerized image scanner. The values were normalized on the basis of the phospholipid content. The results are shown in FIG. **7**, wherein each bar is the average values from three dishes, with error bars corresponding to one standard deviation. In the control, the total protein and GlcCer were 414±47.4 μg/dish and 24.3±1.97 ng/nmol phosphate, respectively.

Approximately 66 and 78% of the GlcCer was lost from the cells treated by 11.3 nM D-threo-4'-hydroxy-P4 and D-threo-3',4'-ethylenedioxy-P4 respectively (FIGS. **7**, **14** and **15**). By contrast, only 27 percent depletion of GlcCer occurred in cells exposed to D-threo-P4 (FIG. **13**). A low level of GlcCer persisted in the cells treated with 113 or 1130 nM of either compound. This may be due to the contribution, by degradation, of more highly glycosylated sphingolipids

or the existence of another GlcCer synthase that is insensitive to the inhibitor.

On the other hand, there was little difference in the total protein content between untreated and treated cells with 11.3 or 113 nM D-threo-4'-hydroxy-P4 and D-threo-3',4'-ethylenedioxy-P4 (FIGS. **14** and **15**). A significant decrease in total protein was observed in the cells treated with 1130 nM of either P4 homologue. In addition, the level of ceramide in the cells treated with 1130 nM D-threo-3',4'-ethylenedioxy-P4 and D-threo-(4'-hydroxy)-P4 was two times higher than that measured in the untreated cells (Table 9). There was no change in ceramide or diradylglycerol levels in cells treated with 11.3 nM or 113 nM concentrations of either compound. Similar patterns for GlcCer levels and protein content were observed at 48 h incubations.

The phospholipid content was unaffected at the lower concentrations of either D-threo-3',4'-ethylenedioxy-P4 or D-threo-(4'-hydroxy)-P4. The ratios of cell protein to cellular phospholipid phosphate (mg protein/nmol phosphate) were 4.94±0.30, 5.05±0.21, 4.84±0.90, and 3.97±0.29 for 0, 11.3, 113, and 1130 nM D-threo-3',4'-ethylenedioxy-P4 respectively, and 4.52±0.39, 4.35±0.10, and 3.68±0.99 for 11.3, 113, and 1130 nM D-threo-4'-hydroxy-P4 suggesting that the changes in GlcCer content were truly related to inhibition of GlcCer synthase activity. These results strongly indicate that the inhibitors D-threo-4'-hydroxy-P4 and D-threo-3',4'-ethylenedioxy-P4, are able to potently and specifically inhibit GlcCer synthesis in intact cells at low nanomolar concentrations without any inhibition of cell growth.

SPECIFIC EXAMPLE 3

Compositions within the scope of invention include those comprising a compound of the present invention in an effective amount to achieve an intended purpose. Determination of an effective amount and intended purpose is within the skill of the art. Preferred dosages are dependent for example, on the severity of the disease and the individual patient's response to the treatment.

As used herein, the term "pharmaceutically acceptable salts" is intended to mean salts of the compounds of the present invention with pharmaceutically acceptable acids, e.g., inorganic acids such as sulfuric, hydrochloric, phosphoric, etc. or organic acids such as acetic.

Pharmaceutically acceptable compositions of the present invention may also include suitable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which may be used pharmaceutically. Such preparations can be administered orally (e.g., tablets, dragees and capsules), rectally (e.g., suppositories), as well as administration by injection.

The pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, e.g., using the conventional mixing, granulating, dragee-making, dissolving or lyophilizing processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding a resulting mixture and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

Suitable excipients are, in particular, fillers such as sugars, e.g., lactose or sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, e.g., tricalcium diphosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, e.g., maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose and/or polyvinylpyrrolidone. If desired, disintegrating agents may be added such as the above-mentioned starches and also carboxymethyl starch, cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are, above all, flow-regulating agents and lubricants, e.g., silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate, and/or polyethylene glycol. Dragee cores are provided with suitable coatings which, if desired, are resistant to gastric juices. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvent or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations, such as acetylcellulose phthalate or hydroxypropylmethyl cellulose phthalate, are used. Dyestuffs or pigments may be added to the tablets or dragee coatings, e.g., for identification or in order to characterize different combinations of active compound doses.

Other pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules may contain the active compounds in the form of granules which may be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be used.

Possible pharmaceutical preparations which can be used rectally include, e.g., suppositories, which consist of a combination of the active compounds with a suppository base. Suitable suppository bases are, e.g., natural or synthetic triglycerides, paraffin hydrocarbons, polyethylene glycols or higher alkanols. It is also possible to use gelatin rectal capsules which consist of a combination of the active compounds with a base. Possible base materials include, e.g., liquid triglycerides, polyethylene glycols or paraffin hydrocarbons.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, e.g., water-soluble salts. In addition, suspension of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, such as sesame oil, or synthetic fatty acid esters, e.g., ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension such as sodium carboxymethylcellulose, sorbitol and/or dextran. Optionally, the suspension may also contain stabilizers.

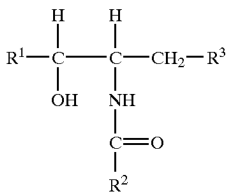
Alternatively, the active compounds of the present invention may be administered in the form of liposomes, pharmaceutical compositions wherein the active compound is contained either dispersed or variously present in corpuscles consisting of aqueous concentrate layers adherent to hydrophobic lipidic layer. The active compound may be present both in the aqueous layer and in the lipidic layer or in the non-homogeneous system generally known as a lipophilic suspension.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention.

All publications cited herein are expressly incorporated by reference.

What is claimed is:

1. A compound of formula I:



a stereoisomer, a pharmaceutically acceptable salt or a mixture thereof, wherein

R^1 is a phenyl, a substituted phenyl group, a branched aliphatic group, or a 7–15 carbons long alkyl chain or a 7–15 carbons long alkenyl chain with a double bond next to the kernel;

R^2 is an alkyl group 6, 7, or 8 carbons long; and

R^3 is a pyrrolidine, azetidine, morpholine or piperidine, in which the nitrogen atom is attached to the kernel.

2. The compound of claim **1**, wherein R^1 is a phenyl group substituted with a functional group selected from the group consisting of a p-methoxy, hydroxyl, methylenedioxy, ethylenedioxy, trimethylenedioxy and cyclohexyl.

3. A method for inhibiting the growth of cancer cells in a patient, comprising the step of administering to the patient a therapeutically effective amount of a composition comprising the compound of any of claim **1** or **2**, wherein said cancer cells are sensitive to said compound.

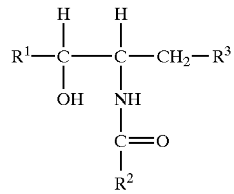
4. A method for treating a patient having a drug resistant tumor, comprising the step of administering to the patient a therapeutically effective amount of a composition comprising the compound of any of claim **1** or **2**, wherein the cells of the tumor are sensitive to said compound.

5. A method for reducing tumor angiogenesis in a patient, comprising the step of administering to the patient a therapeutically effective amount of a composition comprising the compound of any of claim **1** or **2**, wherein said angiogenesis is sensitive to said compound.

6. A method for treating a patient having a glyxosphingolipidosis disorder, comprising the step of administering to the patient a therapeutically effective amount of a composition comprising the compound of any of claim **1** or **2**, wherein the glycosphingolipidos disorder is associated with the presence of glucosylceramide.

7. The method of claim **6**, wherein said glycosphingolipidos disorder is selected from the group consisting of Gaucher disease, Fabry disease, TaySachs, Sandhoff disease, and GM1 gangliosidosis.

8. A compound of formula I:



or a stereoisomer, pharmaceutically acceptable salt, or a mixture thereof, wherein

R^1 is a substituted or unsubstituted phenyl group, a branched aliphatic group, or a 7–15 carbons long alkyl chain or a 7–15 carbons long alkenyl chain with a double bond next to the kernel;

R^2 is an alkyl group 6, 7, or 8 carbons long; and

R^3 is pyrrolidine, in which the nitrogen atom is attached to the kernel.

9. The compound of claim **8**, wherein R^1 is a phenyl group substituted with a functional group selected from the group consisting of a p-methoxy, hydroxyl, methylenedioxy, ethylenedioxy, trimethylenedioxy and cyclohexyl.

10. A method for inhibiting the growth of cancer cells in a patient, comprising the step of administering to the patient a therapeutically effective amount of the compound of claim **8**, wherein said cancer cells are sensitive to said compound.

11. A method for treating a patient having a drug resistant tumor, comprising the step of administering to the patient a therapeutically effective amount of a composition comprising the compound of claim **8**, wherein the cells of the tumor are sensitive to said compound.

12. A method for reducing tumor angiogenesis in a patient, comprising the step of administering to the patient a therapeutically effective amount of a composition comprising the compound of claim **8**, wherein said angiogenesis is sensitive to said compound.

13. A method for treating a patient having a glycosphingolipidosis disorder, comprising the step of administering to the patient a therapeutically effective amount of a composition comprising the compound of claim **8**, wherein the glycosphingolipidosis disorder is associated with the presence of glucosylceramide.

14. The method of claim **13**, wherein said glycosphingolipidosis disorder is selected from the group consisting of Gaucher disease, Fabry disease, TaySachs, Sandhoff disease, and GM1 gangliosidosis.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,916,802 B2
DATED : July 12, 2005
INVENTOR(S) : James A. Shayman et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 33,
Lines 30, 35, 40 and 45, delete "claim" and insert -- claims --.
Lines 42-43, delete "glyxosphingolipidosis" and insert -- glycosphingolipidosis --.
Lines 46, 48 and 49, delete "glycosphingolipidos" and insert
-- glycosphingolipidosis --.

Signed and Sealed this

Eleventh Day of October, 2005

Jon W. Dudas

JON W. DUDAS
Director of the United States Patent and Trademark Office

EXHIBIT B

US007196205B2

(12) **United States Patent**
Siegel et al.

(10) **Patent No.: US 7,196,205 B2**
(45) **Date of Patent: *Mar. 27, 2007**

(54) **SYNTHESIS OF UDP-GLUCOSE: *N*-ACYLSPHINGOSINE GLUCOSYLTRANSFERASE INHIBITORS**

(75) Inventors: **Craig Siegel**, Woburn, MA (US); **James A. Shayman**, Ann Arbor, MI (US); **Carol A. Nelson**, Westford, MA (US); **David J. Harris**, Lexington, MA (US); **Diane P. Copeland**, North Billerica, MA (US)

(73) Assignees: **The Regents of the University of Michigan**, Ann Arbor, MI (US); **Genzyme Corporation**, Cambridge, MA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **11/091,836**

(22) Filed: **Mar. 28, 2005**

(65) **Prior Publication Data**

US 2005/0222244 A1 Oct. 6, 2005

Related U.S. Application Data

(60) Continuation of application No. 10/916,824, filed on Aug. 12, 2004, which is a division of application No. 10/197,227, filed on Jul. 16, 2002, now Pat. No. 6,855,830.

(60) Provisional application No. 60/305,814, filed on Jul. 16, 2001.

(51) **Int. Cl.**
C07D 405/06 (2006.01)

(52) **U.S. Cl.** **548/526**

(58) **Field of Classification Search** 548/526
See application file for complete search history.

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Primary Examiner—Rebecca Anderson
(74) *Attorney, Agent, or Firm*—Hamilton, Brook, Smith & Reynolds, P.C.

(57) **ABSTRACT**

Disclosed is a novel enantiomeric synthesis ceramide-like inhibitors of UDP-glucose: N-acylsphingosine glucosyltransferase. Also disclosed are novel intermediates formed during the synthesis.

16 Claims, 4 Drawing Sheets

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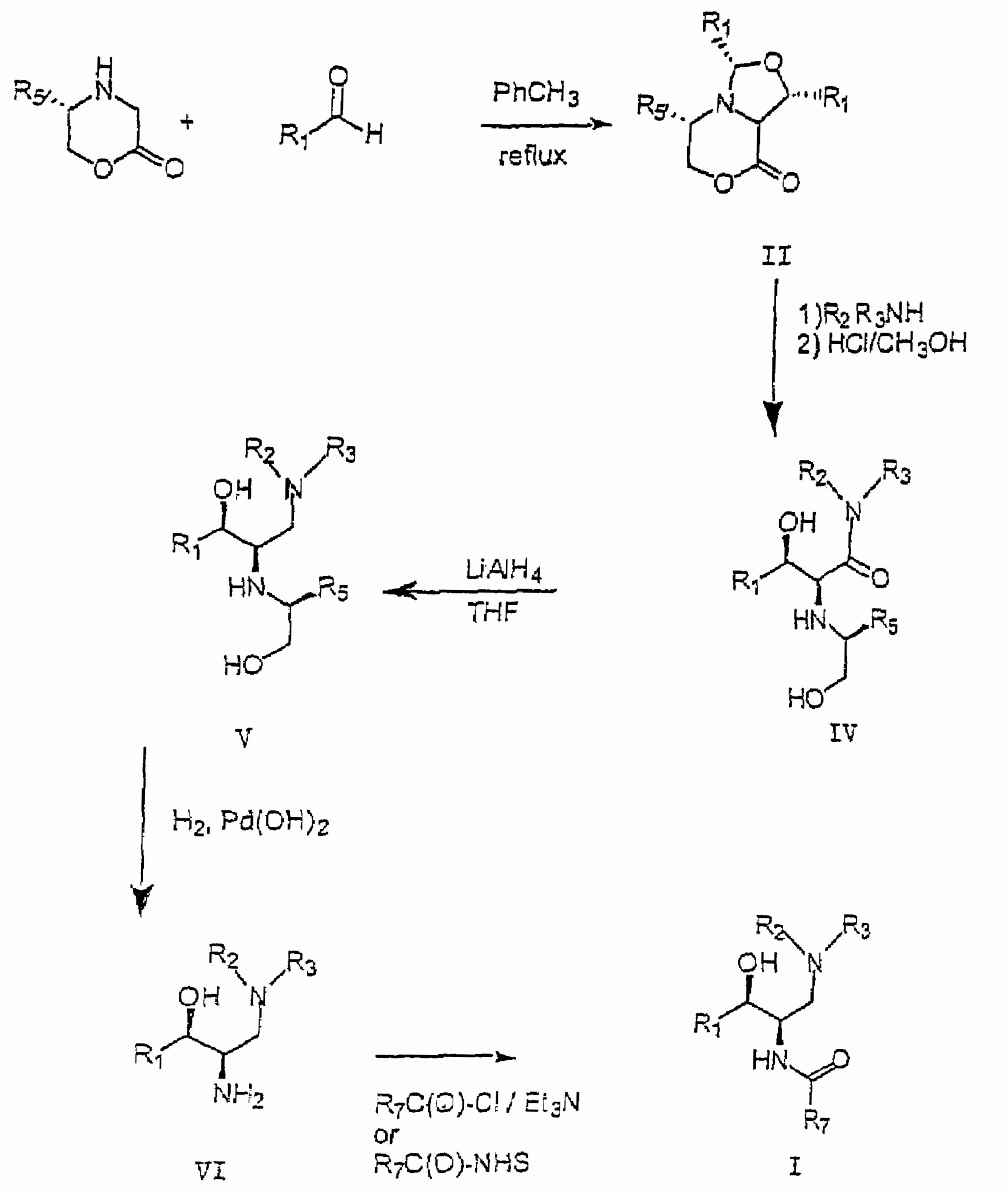

PhCH3
reflux
II
1)R2R3NH
2) HCl/CH3OH
LiAlH4
THF
IV
V
H2, Pd(OH)2
VI
R7C(O)-Cl / Et3N
or
R7C(O)-NHS
I

Figure 1

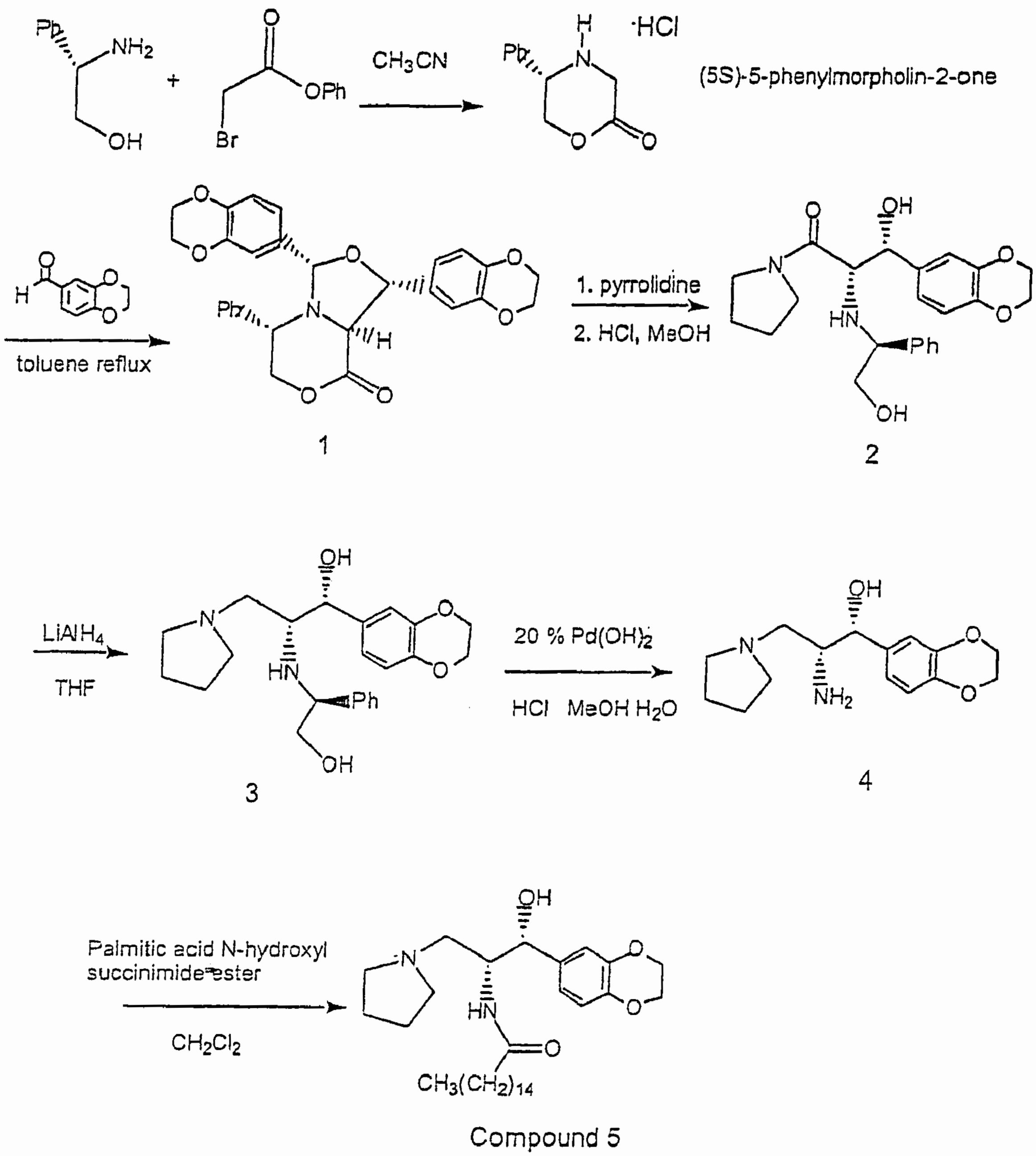
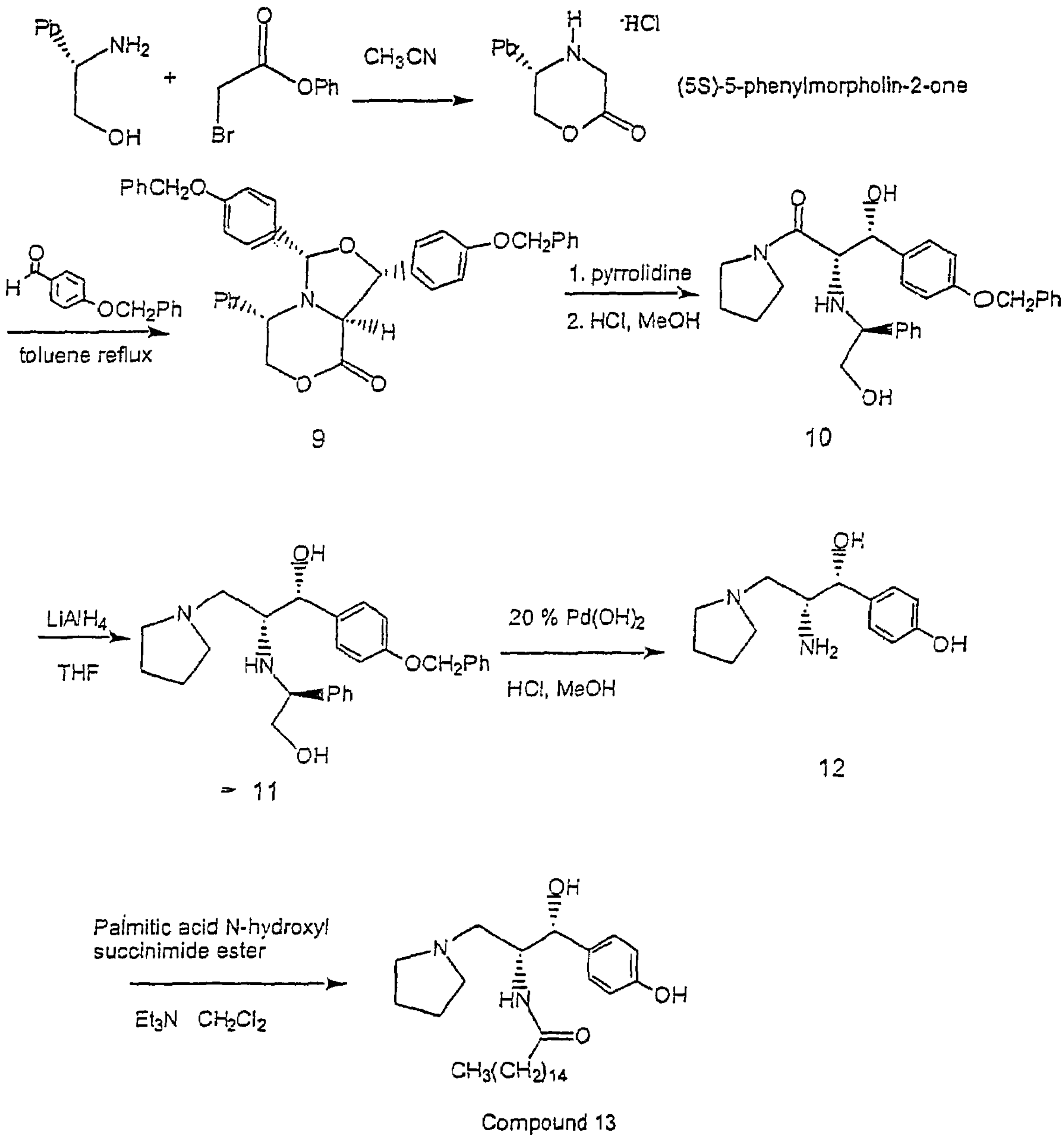
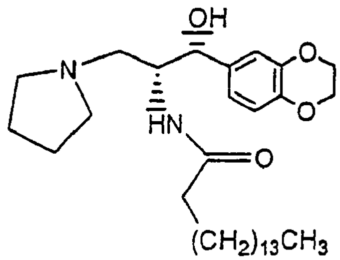

CH3CN
·HCl
(5S)-5-phenylmorpholin-2-one
toluene reflux
1
1. pyrrolidine
2. HCl, MeOH
2
LiAlH4
THF
3
20 % Pd(OH)2
HCl MeOH H2O
4
Palmitic acid N-hydroxyl succinimide ester
CH2Cl2
CH3(CH2)14
Compound 5

Figure 2

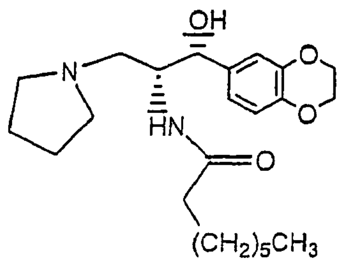


Ph
NH2
OH
O
OPh
Br
CH3CN
H
N
·HCl
O
(5S)-5-phenylmorpholin-2-one
PhCH2O
OCH2Ph
H
toluene reflux
9
1. pyrrolidine
2. HCl, MeOH
10
LiAlH4
THF
11
20 % Pd(OH)2
HCl, MeOH
NH2
12
Palmitic acid N-hydroxyl succinimide ester
Et3N CH2Cl2
CH3(CH2)14
Compound 13

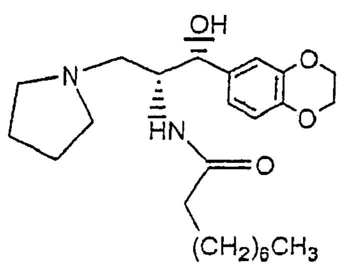
Figure 3



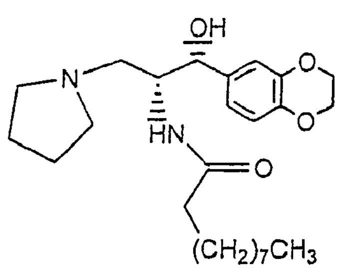
Compound 5



Compound 6



Compound 7



Compound 8

Figure 4

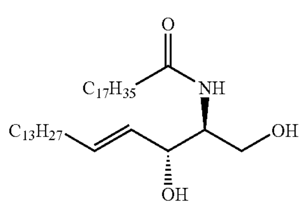
SYNTHESIS OF UDP-GLUCOSE: *N*-ACYLSPHINGOSINE GLUCOSYLTRANSFERASE INHIBITORS

RELATED APPLICATIONS

This application is a continuation U.S. patent application Ser. No. 10/916,824, filed Aug. 12, 2004, which is a divisional of U.S. patent application Ser. No. 10/197,227, filed Jul. 16, 2002, now U.S. Pat. No. 6,855,830, which claims the benefit of U.S. Provisional Application No. 60/305,814, filed Jul. 16, 2001. The entire teachings of these applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Glycosphingolipids (GSLs) are a class of naturally occurring compounds which have a multitude of biological functions, including the ability to promote cell growth, cell differentiation, adhesion between cells or between cells and matrix proteins, binding of microorganisms and viruses to cells, and metastasis of tumor cells. GSLs are derived from glucosylceramide (GlcCer), which is produced from ceramide and UDP-glucose by the enzyme UDP-glucose: N-acylsphingosine glucosyltransferase (GlcCer synthase). The structure of ceramide is shown below:



Ceramide

The accumulation of GSLs has been linked to a number of diseases, including Tay-Sachs, Gaucher's, and Fabry's diseases (see, for example, U.S. Pat. No. 6,051,598). GSLs have also been linked to certain cancers. For example, it has been found that certain GSLs occur only in tumors or at abnormally high concentrations in tumors; exert marked stimulatory or inhibitory actions on tumor growth when added to tumor cells in culture media; and inhibit the body's normal immunodefense system when shed by tumors into the surrounding extracellular fluid. The composition of a tumor's GSLs changes as the tumors become increasingly malignant and antibodies to certain GSLs inhibit the growth of tumors.

Compounds which inhibit GlcCer synthase can lower GSL concentrations and have been reported to be useful for treating a subject with one of the aforementioned diseases. A number of potent inhibitors of GlcCer, referred to herein as "amino ceramide-like compounds", are disclosed in U.S. Pat. Nos. 6,051,598, 5,952,370, 5,945,442, 5,916,911 and 6,030,995. The term "ceramide-like compounds" refers to analogs of ceramide in which: 1) the primary alcohol is replaced with a substituted amino group; and 2) the alkenyl group is replaced with an aryl group, preferably phenyl or substituted phenyl. The corresponding N-deacylated compounds are referred to as "sphingosine-like compounds."

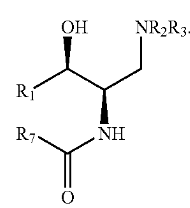
Unfortunately, known methods of preparing amino ceramide-like compounds are poorly suited for manufacturing on an industrial scale. Because of the two chiral centers, most known syntheses generate four diastereoisomers, resulting in the need to separate diastereomers by chromatography and to isolate the desired enantiomer by crystallization after derivitization with optically active reagents, e.g., dibenzoyltartaric acid isomers (see, for example, Inokuchi and Radin, *Journal of Lipid Research* 28: 565 (1987)). Neither of the processes are amenable to large scale preparations. Enantioselective synthesis of amino ceramide-like compounds using diastereoselective reductions have been reported (Mitchell, et al., *J. Org. Chem.* 63:8837 (1998) and Nishida, et al., *SYNLETT* 1998: 389 (1998)), but require over ten steps, some of which utilized expensive reagents such as diisobutylaluminum hydride (DIABAL) and Garner Aldehyde (tert-butyl (R)-(+)-4 formyl-2,2-dimethyl-3-oxazolidine carboxylate). Thus, there is a critical need for enantioselective syntheses of amino ceramide-like compounds which are more economical and efficient, and involve fewer steps than known syntheses.

SUMMARY OF THE INVENTION

Provided herein is an efficient, highly enantioselective synthesis of amino ceramide-like compounds. This synthesis of amino ceramide-like compounds involves only five steps from known compounds. For example, the ceramide-like compound designated as "Compound 5" in FIG. **2** was produced in an enantiomeric excess of at least 99.6% and an overall yield of 9% (see Examples 1 and 2). Novel intermediates prepared during the course of the synthesis are also disclosed.

The present invention is directed is a method of preparing a ceramide-like compound represented by Structural Formula (I):

(I)



R_1 is a substituted or unsubstituted aromatic group; preferably, R_1 is a substituted or unsubstituted phenyl group, more preferably phenyl substituted in the meta/para positions with —OCH_2O—, —OCH_2CH_2O— or in the para position with halo, lower alkyl thiol, —OH, —O(phenyl), —OCH_2(phenyl), lower alkyl, amino, lower alkyl amino, lower dialkyl amino, or —O(lower alkyl);

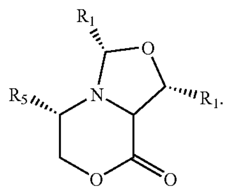
R_2 and R_3 are independently —H, a substituted or unsubstituted aliphatic group or, taken together with the nitrogen atom to which they are bonded, are a substituted or unsubstituted non-aromatic heterocyclic ring.

R_7 is a substituted or unsubstituted aliphatic group, preferably a C1–C30 straight chain unsubstituted aliphatic group or a C1–C30 straight chained aliphatic group substituted with one or more C1–C2 alkyl groups, more preferably an unsubstituted C1–C30 straight chain alkyl or alkenyl group

and even more preferably an unsubstituted C7–C10 or C10–C16 straight chain alkyl or alkenyl group.

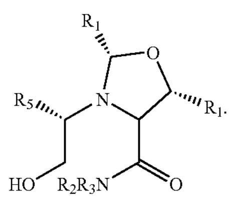
The method of preparing a ceramide-like compound represented by Structural Formula (I) comprises a first step whereby an amine compound HNR_2R_3 is reacted with a cyclic starting material represented by Structural Formula (II):

(II)



The reaction between the amine compound HNR_2R_3 and the cyclic starting material represented by Structural Formula (II) forms an amide intermediate represented by Structural Formula (III):

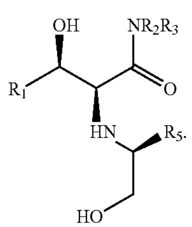
(III)



In Structural Formulas (II) and (III), R_1–R_3 are as described for Structural Formula (I); and R_5 is a substituted or unsubstituted aromatic group, preferably a substituted or unsubstituted phenyl group.

The method of preparing a ceramide-like compound represented by Structural Formula (I) comprises a second step whereby the amino acetal group in the intermediate represented by Structural Formula (III) is hydrolyzed to form the acyclic compound represented by Structural Formula (IV).

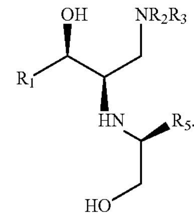
(IV)



R_1, R_2, R_3 and R_5 in Structural Formulas (IV) are as defined in Structural Formulas (I)–(III).

The method of preparing a ceramide-like compound represented by Structural Formula (I) comprises a third step whereby the acyclic precursor compound represented by Structural Formula (IV) is reacted with an amide reducing agent to form a compound represented by Structural Formula (V):

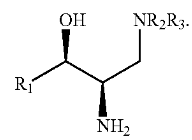
(V)



R_1, R_2, R_3 and R_5 in Structural Formula (V) are as defined in Structural Formulas (I)–(IV).

The method of preparing a ceramide-like compound represented by Structural Formula (I) comprises a fourth step whereby the —NHCH(—CH_2OH)R_5 group of the amine compound represented by Structural Formula (V) is debenzylated to form a sphingosine-like compound represented by Structural Formula (VI):

(VI)



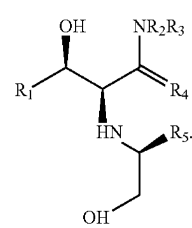
Preferably, the debenzylation is achieved by hydrogenation. R_1, R_2 and R_3 are as described for Structural Formulas (I)–(V).

The method of preparing a ceramide-like compound represented by Structural Formula (I) comprises a fifth step whereby the sphingosine-like compound represented by Structural Formula (VI) is acylated to form the ceramide-like compound represented by Structural Formula (I).

Other embodiments of the present invention include each of the individual reactions described above, taken separately and in combination with the other reactions.

Other embodiments of the present invention are intermediates in the preparation of the ceramide-like compound represented by Structural Formula (I) by the methods disclosed herein. In one example, the present invention is directed to an intermediate represented by Structural Formula (VII):

(VII)

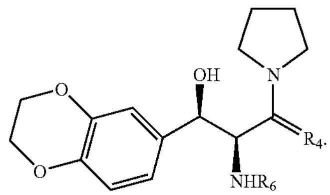


R_1–R_3 and R_5 are as described above for Structural Formulas (I)–(VI); and

R_4 is H_2 or O.

In another embodiment, the present invention is directed to an intermediate represented by Structural Formula (VIII):

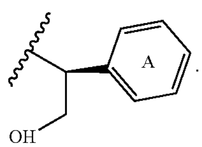
(VIII)



R_4 is H_2 or O; and

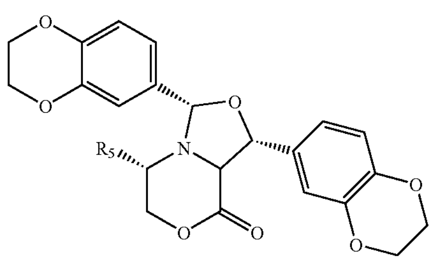
R_6 is represented by Structural Formula (IX):

(IX)



Phenyl ring A in Structural Formula (IX) is substituted or unsubstituted. Preferably, however, phenyl ring A is unsubstituted. Alternatively, R_4 in Structural Formula (VIII) is H_2 and R_6 is —H.

In another embodiment, the present invention is directed to an intermediate represented by Structural Formula (X):



R_5 in Structural Formula (X) is as defined for Structural Formula (I).

The methods of the present invention can be utilized to prepare ceramide-like compounds that inhibit the enzyme GlcCer synthase in five steps from known starting materials. The synthesis is highly efficient, resulting in an overall yield that is generally greater than 8% and in an enantiomeric excess that is typically greater than 99%. The synthesis utilizes inexpensive reagents and therefore provides an economical route to potent inhibitors of GlcCer synthase.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic showing the synthesis of ceramide-like compounds represented by Structural Formula (I) using the methods and intermediates disclosed herein.

FIG. **2** is a schematic showing the synthesis of ceramide-like Compound (5) using the methods disclosed herein.

FIG. **3** is a schematic showing the synthesis of ceramide-like compound (13) using the methods disclosed herein.

FIG. **4** shows the structures of Compounds (5)–(8).

DETAILED DESCRIPTION OF THE INVENTION

Described herein is a five step synthesis of amino ceramide-like compounds from known starting materials. The synthesis begins with the preparation of the cyclic starting material represented by Structural Formula (II). The cyclic starting material is reacted with a suitable amine, thereby opening the lactone ring and forming the amide intermediate represented by Structural Formula (III). The amino acetal in the amide intermediate is hydrolyzed to form the acyclic compound represented by Structural Formula (IV). The amide of this acyclic compound is reduced with an amide reducing agent to form an amine compound represented by Structural Formula (V), which is in turn debenzylated to form the sphingosine-like compound represented by Structural Formula (VI). The primary amine of the sphingosine-like compound represented by Structural Formula (VI) can then be acylated to form an amino ceramide-like compound. This synthesis is shown schematically in FIG. **1**. A detailed description of each reaction in the synthesis is provided below.

The cyclic starting material represented by Structural Formula (II) is prepared according to methods described in Alker, et al., *Tetrahedron* 54:6089 (1998) and Harwood and Robertson, *Chem. Commun.* 1998:2641 (1998). Specifically, (5S)-5-phenylmorpholin-2-one is reacted with at least two equivalents and preferably from about 2.5 to about 5.0 equivalents of aryl aldehyde R_1CHO under dehydrating conditions. R_1 is as defined in Structural Formula (I). "Dehydrating conditions" refer to conditions under which water is removed from the reaction mixture. Removal of water can be achieved, for example, by carrying out the reaction in presence of a reagent (a "dehydrating reagent") that reacts with water (e.g., molecular sieves) but is substantially inert towards the other reagents present in the reaction mixture, or removal of water can also be achieved by azeotroping with a solvent such as toluene. Sufficient dehydrating reagent is used to remove the two equivalents of water (relative to cyclic starting material) released during the reaction. The concentration of reagents if typically between about 0.01 M and about 5.0 M, more typically between about 0.1 M and about 1.0 M; suitable reaction temperatures range between about 50° C. and about 150° C., preferably between about 100° C. and about 120° C.

The cyclic starting material is converted to the amide intermediate represented by Structural Formula (II) by reacting the cyclic starting material with the amine NHR_2R_3 under conditions suitable for amidating an ester with an amine. Such conditions are well known in the art and are described, for example, in March, "Advanced Organic Chemistry—Reactions, Mechanisms and Structure", Third Edition, John Wiley & Sons, 1985, pages 375–76, and references cited therein. Although an excess of either reagent can be used, cyclic starting material is more commonly the limiting reagent. Generally up to about fifteen equivalents of amine relative to cyclic starting material are used, typically up to about eight equivalents. The reaction can be done neat, however, it is more usually carried out in a aprotic, non-nucleophilic solvent at amine concentrations as dilute as 0.01 M. Amine concentrations are more typically, however, between about 0.4 M and about 4.0 M. Suitable solvents include halogenated solvents such as chloroform, dichloromethane and 1,2-dichloroethane, acetonitrile, dimethylformamide (DMF), ethereal solvents such as diethyl ether, tetrahydrofuran (THF) and 1,4-dioxane and aromatic solvents such as benzene and toluene. Suitable reaction tem-

peratures generally range from about 0° C. to about 100° C., typically between about 25° C. to about 35° C.

Conditions for hydrolyzing aminoacetals are known in the art and are described, for example, in March, "Advanced Organic Chemistry—Reactions, Mechanisms and Structure", Third Edition, John Wiley & Sons, 1985, pages 329–32, and references cited therein. For example, the aminoacetal group in the amide intermediate represented by Structural Formula (III) can be hydrolyzed with dilute aqueous mineral acid. Suitable acids include hydrochloric acid, sulfuric acid or phosphoric acid, although hydrochloric is the most common choice. Organic acids such as acetic acid and sulfonic acids (e.g., methansulfonic acid, toluenesulfonic acid, trifluormethylsulfonic acid and the like) can also be used. At least one equivalent of acid relative to the intermediate is typically used, but an excess of acid is preferred to ensure complete hydrolysis, for example, excesses of at least ten fold, preferably an excess of about two to about three fold and more preferably between about 10–50%. The concentration of acid in the reaction mixture is generally between about 0.05 M to about 1.0 M, typically between about 0.1 M and about 0.5 M. An organic co-solvent miscible with water is often used to solubilize the intermediate. Examples include alcohols such as methanol or ethanol and DMF. Common solvent ratios of organic solvent to water range between about 1:1 to about 8:1. Suitable reaction temperatures range from ambient temperature to about 100° C., preferably between about 60° C. to about 80° C. Alternatively, the amino acetal can be hydrolyzed with Lewis acids such as trimethylsilyl iodide, wet silica gel or $LiBF_4$ in wet acetonitrile, as described in March, supra.

An "amide reducing agent" is a reagent which can reduce an amide to an amine. Such reagents are known in the art and are disclosed in, for example, in March, "Advanced Organic Chemistry—Reactions, Mechanisms and Structure", Third Edition, John Wiley & Sons, 1985, pages 1099–1100, Brown and Krishnamurthy, *Aldrichimica Acta* 12:3 (1979) and references cited therein. Examples include lithium aluminum hydride, lithium triethyl borohydride, borane reagents (e.g., borane.tetrahydrofuran, borane.methyl sulfide, disiamylborane, and the like), aluminum hydride, lithium trimethoxy aluminum hydride and triethyloxonium fluoroborate/sodium borohydride. In the method of the present invention, lithium aluminum hydride is the most commonly used amide reducing agent. Although as little as 0.5 equivalents of lithium aluminum hydride relative to amide starting material can be used, it is more common to use an excess, often up to about five equivalents. Preferably, between about 1.5 and about 2.5 equivalents of lithium aluminum hydride are used relative to amine starting material. Ethereal solvents are typically used for the reduction; examples include diethyl ether, THF, glyme, diglyme and 1,4-dioxane. Suitable concentrations of reducing agent are generally between about 0.1 M and about 5.0 M, more typically between about 0.8 M and about 1.5 M. The reduction is most commonly carried out at ambient temperature, but temperatures between about 0° C. and about 80° C. or 100° C. can also be used.

To form the sphingosine-like compound represented by Structural Formula (VI), the amine compound represented by Structural Formula (V) is debenzylated. The term "debenzylating" is used herein to refer to cleaving the carbon-nitrogen bond of a group $—NH—CH_2Z$, wherein Z is an aryl group, preferably phenyl. Optionally, the methylene group can be replaced with a methine group. With respect to the sphingosine-like compound represented by

Structural Formula (VI), "debenzylation" refers to converting the $—NHCH(—CH_2OH)R_5$ group to $—NH_2$, Debenzylation conditions are well known in the art and are disclosed, for example, in Greene and Wuts, "Protective Groups in Organic Synthesis", John Wiley & Sons (1991), pages 384–86 and references cited therein.

Preferably, debenzylation is achieved by hydrogenation under a hydrogen atmosphere and in the presence of a hydrogenation catalyst. Suitable hydrogen pressures are generally between about atmospheric pressure and about 1000 pounds per square inch. Other sources of hydrogen (e.g., formic acid, ammonium formate, cyclohexene and the like) can also be used. Suitable hydrogenation catalysts include 20% palladium hydroxide on carbon (Perlman's catalyst), palladium chloride, palladium, platinum oxide and palladium on carbon. Typically, between about 10% and about 100% weight/weigh (w/w) relative to amine compound is used. In most instances, an organic acid such as formic acid, acetic acid or trifluoroacetic acid or an inorganic acid such as hydrochloric acid or sulfuric acid is present, for example, between about one to about five equivalents relative to amine compound, preferably between about 1.6 to about 2.4 equivalents. The reaction is most commonly carried out in an alcoholic solvent such as methanol or ethanol with water as co-solvent (e.g., between 0% and about 50% volume/volume (v/v), preferably between about 5% and about 15% v/v). Reaction temperatures between about 0° C. and about 50° C. are suitable, preferably between about 25° C. and about 40° C.

Many debenzylation conditions other than hydrogenation are known in the art and are included in the present invention. Examples include sodium metal and NH_3 (see, for example, du Vigneaud and Behrens, *J. Biol. Chem.* 117:27 (1937)), CCl_3CH_2OCOCl, CH_3CN (see, for example, Rawal, et al., *J. Org. Chem.,* 52:19 (1987)), $Me_3SiCH_2CH_2OCOCl$, THF, −50° C., then 25° C. overnight (see, for example, Campbell, et al., *Tetrahedron Lett.,* 28:2331 (1987)), α-chloroethyl chloroformate and sodium hydroxide (see, for example Olofson, et al., *J. Org. Chem.* 49:2081 (1984) and DeShong and Kell, *Tetrahedron Lett.,* 27:3979 (1986)), vinyl chloroformate (see, for example, Olofson et al., *Tetrahedron Lett.,* 1977:1567 (1977) and Cooley and Evain, *Synthesis,* 1989:1 (1989)), RuO_4, NH_3, H_2O (see, for example, Gao and Jones, *J. Am. Chem. Soc.,* 109:1275 (1987)) and m-chloroperoxybenzoic acid followed by $FeCl_2$, −10° C. (see, for example, Monkovic, et al., *Synthesis,* 1985:770 (1985).

The sphingosine-like compound represented by Structural Formula (VI) is converted to a ceramide-like compound by acylating the free amine. Acylations of amine groups are well known in the art and can be carried out, for example, by reacting the amine with an acylating agent $R_7C(O)—X$. R_7 is as described above for Structural Formula (I) and X is a leaving group that is readily displaced by a primary amine. Conditions for this reaction are described in, for example, in March, "Advanced Organic Chemistry—Reactions, Mechanisms and Structure", Third Edition, John Wiley & Sons, 1985 and references cited therein. Examples of suitable acylating agents include acid halides, anhydrides or esters. Preferably, the amine is acylated with an acid chloride. Generally, equimolar amounts of the sphingosine-like compound and the acid chloride are used in the presence of a small excess, relative to the acid chloride, of a tertiary amine such as triethylamine, diisopropylethylamine, dimethylaminopyridine or pyridine is used. However, an excess of acid chloride (typically about 10–50%) can be used when the sphingosine-like compound is limiting, and vice versa. The

concentrations of the reagents in the reaction mixture normally vary between about 0.005 M and about 5.0 M, and are preferably between about 0.05 M and about 0.5 M. The excess of amine base can be greater than about 100%, but is typically between about 5% and about 25%. Aprotic solvents such as halogenated solvents are preferred (e.g., chloroform, methylene chloride and 1,2-dichloromethane), however other aprotic solvents such as ethereal solvents and hydrocarbon solvents can be suitable substitutes. Ambient temperature is normally preferred for the reaction, but temperatures between about 0° C. and about 50° can also be used.

Alternatively, the acylating agent is an activated ester $R_7C(O)$—OX', wherein —OX' is readily displaced by a primary amine. Methods of acylating an amine with activated esters are known in the art and are described in, for example, March, "Advanced Organic Chemistry—Reactions, Mechanisms and Structure", Third Edition, John Wiley & Sons, 1985, pages 371–375, and references cited therein. Many activated esters are stable enough to be isolated. N-Hydroxy succinimidyl esters, some of which are commercially available from Aldrich Chemical Co., Milwaukee, Wis., are one example of activated esters of this type. Conditions suitable for forming an amide with an acid chloride acylating agent, described in the prior paragraph, can typically be used with a stable activated ester. In contrast with acid chlorides, which require activation with tertiary amines, activated esters are reactive enough so that they form amides directly in the presence of primary amines. Therefore, the tertiary amine can be omitted from the acylation reaction when activated esters are used.

Alternatively, an activated ester is formed in situ. Formation of an activated ester in situ requires a "coupling agent", which is a reagent that replaces the hydroxyl group of a carboxyl acid with a group which is susceptible to nucleophilic displacement. Examples of coupling agents include 1,1'-carbonyldiimidazole (CDI), isobutyl chloroformate, dimethylaminopropylethyl-carbodiimide (EDC), dicyclohexyl carbodiimide (DCC). When amidating by in situ generation of an activated ester, an excess of either the carboxylic acid or amine can be used (typically a 50% excess, more typically about a 10–15% excess). However, it is more common when carrying out the present invention to use the amine compound as the limiting reagent. Generally, from about 1.0 mole to about 10 moles of coupling agent are used per mole of carboxylic acid, preferably from about 1.0 mole to about 1.5 moles of coupling agent per mole of carboxylic acid. The reaction is generally carried out in aprotic solvents, for example, halogenated solvents such as methylene chloride, dichloroethane and chloroform, ethereal solvents tetrahydrofuran, 1,4-dioxane and diethyl ether and dimethylformamide. Suitable reaction temperatures generally range from between about 0° to about 100° C., but the reaction is preferably carried out at ambient temperature.

Examples of specific conditions for carrying out the reactions described herein are provided in Examples 1 and 2.

By utilizing the enantiomer of the compound represented by Structural Formula (II) as the cyclic starting material, the enantiomer of the compounds represented by Structural Formulas (III)–(VI) and (I) can be prepared by utilizing the methods described herein. The enantiomer of the cyclic starting material represented by Structural Formula (III) can be prepared by reacting (5R)-5-phenylmorpholin-2-one with two equivalents of the aldehyde R_1CHO under dehydrating conditions, as described above. The enantiomer of compounds represented by Structural Formula (III), (VII), (VIII) and (X) and methods of preparing the enantiomers of the compounds represented by Structural Formulas (II)–(VI) and (I) using procedures disclosed herein are encompassed within the present invention.

The term "enantiomer" as it used herein, and structural formulas depicting an enantiomer are meant to include the "pure" enantiomer free from its optical isomer as well as mixtures of the enantiomer and its optical isomer in which the enantiomer is present in an enantiomeric excess, e.g., at least 10%, 25%, 50%, 75%, 90%, 95%, 98%, or 99% enantiomeric excess.

With regard to the variables R_1–R_5 in Structural Formulas (I)—(IX), an "aliphatic group" is non-aromatic, consists solely of carbon and hydrogen and may optionally contain one or more units of unsaturation, e.g., double and/or triple bonds. An aliphatic group may be straight chained, branched or cyclic. When straight chained or branched, an aliphatic group typically contains between about 1 and about 30 carbon atoms, more typically between about 1 and about 24 carbon atoms. When cyclic, an aliphatic group typically contains between about 3 and about 10 carbon atoms, more typically between about 3 and about 7 carbon atoms. Aliphatic groups are preferably lower alkyl groups, which include C1–30 straight chained or branched saturated hydrocarbons, preferably C1–C24 straight chained or branched saturated hydrocarbons. Examples include methyl, ethyl, n-propyl, iso-propyl, n-butyl, sec-butyl and tert-butyl.

Aromatic groups include carbocyclic aromatic groups such as phenyl, 1-naphthyl, 2-naphthyl, 1-anthracyl and 2-anthacyl, and heterocyclic aromatic groups such as N-imidazolyl, 2-imidazole, 2-thienyl, 3-thienyl, 2-furanyl, 3-furanyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidy, 4-pyrimidyl, 2-pyranyl, 3-pyranyl, 3-pyrazolyl, 4-pyrazolyl, 5-pyrazolyl, 2-pyrazinyl, 2-thiazole, 4-thiazole, 5-thiazole, 2-oxazolyl, 4-oxazolyl and 5-oxazolyl.

Aromatic groups also include fused polycyclic aromatic ring systems in which a carbocyclic aromatic ring or heteroaryl ring is fused to one or more other heteroaryl rings. Examples include 2-benzothienyl, 3-benzothienyl, 2-benzofuranyl, 3-benzofuranyl, 2-indolyl, 3-indolyl, 2-quinolinyl, 3-quinolinyl, 2-benzothiazole, 2-benzooxazole, 2-benzimidazole, 2-quinolinyl, 3-quinolinyl, 1-isoquinolinyl, 3-quinolinyl, 1-isoindolyl and 3-isoindolyl.

Non-aromatic heterocyclic rings are non-aromatic carbocyclic rings which include one or more heteroatoms such as nitrogen, oxygen or sulfur in the ring. The ring can be five, six, seven or eight-membered. Examples include morpholinyl, thiomorpholinyl, pyrrolidinyl, piperazinyl, piperidinyl, azetidinyl, azacycloheptyl, or N-phenylpiperazinyl.

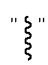
Suitable substituents on a lower alkyl, aliphatic, aromatic, non-aromatic, heterocyclic or benzyl group are those which do not substantially interfere with the reactions described herein. "Interfering with a reaction" refers to substantially decreasing the yield (e.g., a decrease of greater than 50%) or causing a substantial amount of by-product formation (e.g., where by-products represent at least 50% of the theoretical yield). Interfering substituents can be used, provided that they are first converted to a protected form. Suitable protecting groups are known in the art and are disclosed, for example, in Greene and Wuts, "Protective Groups in Organic Synthesis", John Wiley & Sons (1991).

Suitable substituents on an alkyl, aliphatic, aromatic, non-aromatic heterocyclic ring or benzyl group include, for example, halogen (—Br, —Cl, —I and —F), —OR, —CN, —NO_2, —NR_2, —COOR, —$CONR_2$, —SO_kR (k is 0, 1 or 2) and —NH—C(═NH)—NH_2. Each R is independently —H, an aliphatic group, a substituted aliphatic group, a benzyl group, a substituted benzyl group, an aromatic group or a substituted aromatic group, and preferably —H, a lower alkyl group, a benzylic group or a phenyl group. A substituted non-aromatic heterocyclic ring, benzylic group or aromatic group can also have an aliphatic or substituted aliphatic group as a substituent. A substituted alkyl or aliphatic group can also have a non-aromatic heterocyclic ring, benzyl, substituted benzyl, aromatic or substituted aromatic group as a substituent. A substituted alkyl, substituted aliphatic, substituted non-aromatic heterocyclic, substituted aromatic or substituted benzyl group can have more than one substituent.

When R_1 is a substituted phenyl group, examples of preferred substitutents include —OCH_2O—, —OCH_2CH_2O—, halo, (lower alkyl)O—, lower alkyl thiol, lower dialkylamine, —OH, —O(phenyl), —OCH_2 (phenyl), lower alkyl, amine and lower alkyl amino.

When R_5 is a substituted phenyl group, examples of preferred substitutents include halo, (lower alkyl)O—, —O(phenyl) and lower alkyl.

In the structural formulas depicted herein, the remainder of the molecule or compound to which a chemical group or moiety is connected is indicated by the following symbol:



For example, the corresponding symbol in Structural Formula (IX) indicates that the depicted group, which is represented by R_6 in Structural Formula (VIII), is connected via the benzylic carbon to the amine in Structural Formula (VIII) by a single covalent bond.

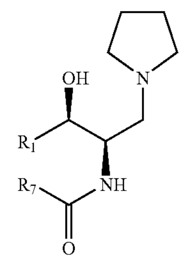
In preferred embodiments of the present invention the variables used herein are defined as follows: R_1 is a substituted or unsubstituted phenyl group; R_2 and R_3 are independently —H, an unsubstituted C1–C5 alkyl group or, taken together with the nitrogen atom to which they are bonded, are an unsubstituted C3–C10 non-aromatic heterocyclic ring; R_5 is a substituted or unsubstituted phenyl group, preferably phenyl; and R_7 is a C1–C30 straight chain unsubstituted aliphatic group or a C1–C30 straight chained aliphatic group substituted with one or more C1–C2 alkyl group and more preferably an unsubstituted C1–C30 straight chain alkyl or alkenyl group.

In another preferred embodiment, —NR_2R_3, taken together, is pyrrolidinyl. More preferably, —NR_2R_3, taken together, is pyrrolidinyl and R_5 is phenyl in compounds comprising R_2, R_3 and R_5. Even more preferably, in compounds comprising R_1, R_2, R_3 and R_5, R_1 is a substituted or unsubstituted phenyl group (preferably phenyl substituted in the meta/para positions with —OCH_2O—, —OCH_2CH_2O— or in the para position with halo, lower alkyl thiol, —OH, —O(phenyl), —O—CH_2(phenyl), lower alkyl, amino, lower alkyl amine, lower dialkyl amino, or —O(lower alkyl), —NR_2R_3, taken together, is pyrrolindinyl and R_5 is phenyl.

In another preferred embodiment, —NR_2R_3, taken together, is piperidyl. More preferably, —NR_2R_3, taken together, is piperidyl and R_5 is phenyl in compounds comprising R_2, R_3 and R_5. Even more preferably, in compounds comprising R_1, R_2, R_3 and R_5, R_1 is a substituted or unsubstituted phenyl group (preferably phenyl substituted in the meta/para positions with —OCH_2O—, —OCH_2CH_2O— or in the para position with halo, lower alkyl thiol, —OH, —O(phenyl), —OCH_2— (phenyl), —OCH_2 (phenyl), lower alkyl, amino, lower alkyl amino, lower dialkyl amino, or —O(lower alkyl), —NR_2R_3, taken together, is piperidyl and R_5 is phenyl.

Examples of ceramide-like compounds which can be prepared by the methods of the present invention are represented by Structural Formula (XI):

(XI)



R_1 is phenyl substituted in the meta/para positions with —OCH_2O— or —OCH_2CH_2O— or in the para position with halo, CH_3O—, CH_3CH_2O—, $CH_3CH_2CH_2O$—, $CH_3(CH_3)CHO$—, CH_3—, CH_3CH_2—, $CH_3CH_2CH_2$—, $CH_3(CH_3)CH$—, —OH or —)CH_2(phenyl); and R_7 is $CH_3(CH_2)_n$— or $CH_3(CH_2)_{n-2}$ CH═CH—, wherein n is an integer from 0 to about 30. Preferably, n is 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24. More preferably, R_1 is phenyl substituted meta/para with —OCH_2CH_2O—.

Compounds of this invention which possess a sufficiently acidic, a sufficiently basic, or both functional groups, and accordingly can react with any of a number of inorganic bases, and inorganic and organic acids, to form a salt. Thus, the present invention also includes salts of the intermediates represented by Structural Formulas (VII), (VIII) and (X). Physiologically acceptable salts are preferred. Acids commonly employed to form acid addition salts are inorganic acids such as hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, phosphoric acid, and the like, and organic acids such as p-toluenesulfonic acid, methanesulfonic acid, oxalic acid, p-bromophenyl-sulfonic acid, carbonic acid, succinic acid, citric acid, benzoic acid, acetic acid, and the like. Examples of such salts include the sulfate, pyrosulfate, bisulfate, sulfite, bisulfite, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, chloride, bromide, iodide, acetate, propionate, decanoate, caprylate, acrylate, formate, isobutyrate, caproate, heptanoate, propiolate, oxalate, malonate, succinate, suberate, sebacate, fumarate, maleate, butyne-1,4-dioate, hexyne-1,6-dioate, benzoate, chlorobenzoate, methylbenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, phthalate, sulfonate, xylenesulfonate, phenylacetate, phenylpropionate, phenylbutyrate, citrate, lactate, gamma-hydroxybutyrate, glycolate, tartrate, methanesulfonate, propanesulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, mandelate, and the like.

Base addition salts include those derived from inorganic bases, such as ammonium or alkali or alkaline earth metal hydroxides, carbonates, bicarbonates, and the like. Such bases useful in preparing the salts of this invention thus include sodium hydroxide, potassium hydroxide, ammonium hydroxide, potassium carbonate, and the like.

The entire teachings of the publications cited in this application are incorporated herein by reference.

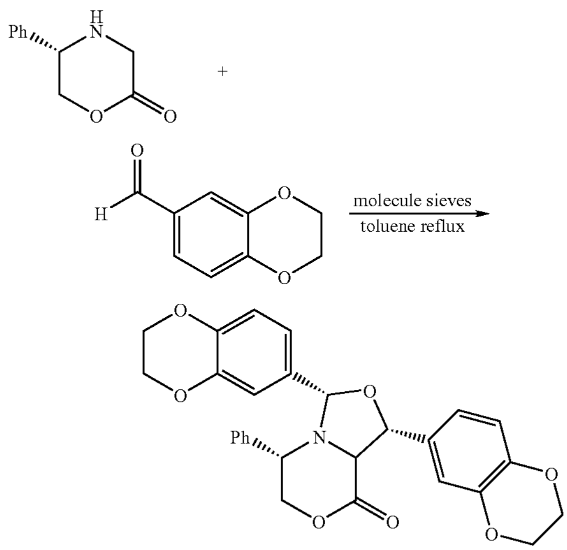
EXEMPLIFICATION

EXAMPLE 1

Small Scale Preparation of Ceramid-Like Compounds

Intermediate 1

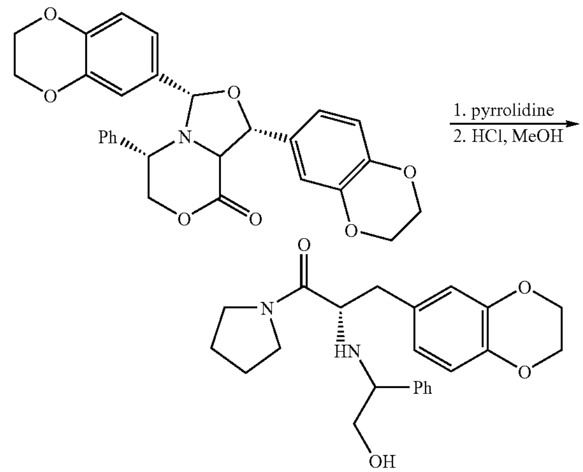
(1R,3S,5S,8aS)-1,3-Bis-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-5-phenyl-tetrahydro-oxazolo[4,3-c][1,4]oxazin-8-one



To a stirred solution of (5S)-5-phenylmorpholim-2-one (2.00 g, 11.3 mmol) (prepared as in: Dellaria, J. F.: Santarsiero, B. D. *J. Org. Chem.*, 1989, 54, 3916) and 1,4-benzodioxan-6-carboxaldehyde (5.56 g, 33.9 mmol) in toluene (125 mL) was added 4 Å molecular sieves (approximately 20 mL). The mixture was heated at reflux for 72 hours, filtered free of sieves and concentrated. The resulting amber gum was flash chromatographed over silica (diethyl ether/hexane) to furnish a pale yellow solid. This material was further purified by trituration with diethyl ether to afford 1.89 g (34%) product as a fluffy white solid: ^{1}H NMR ($CDCl_3$) δ 7.31–7.17 (m, 5H), 6.95–6.79 (m, 5H), 5.32–5.27 (m, 2H), 4.43–4.28 (m, 2H), 4.24 (s, 4H), 4.18 (m, 4H), 4.16–4.08 (m, 2H) ppm.

Intermediate 2

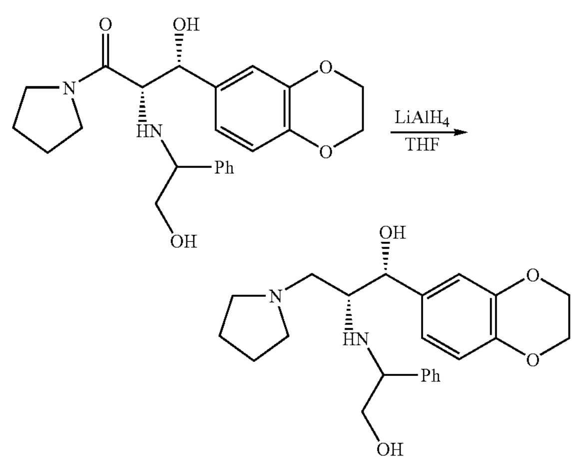
(2S,3R,1"S)-3-(2',3'-Dihydro-benzo[1,4]dioxin-6'-yl)-3-hydroxy-2-(2"-hydroxy-1"-phenyl-ethylamino)-1-pyrrolidin-1-yl-propan-1-one



To a stirred solution of Intermediate 1 (1.80 g, 3.69 mmol) in chloroform (20 mL) was added pyrrolidine (2.0 mL, 24 mmol). The solution was stirred overnight and then concentrated. The resulting colorless tacky foam was taken up in methanol (16 mL) and 1 N hydrochloric acid (4 mL). The mixture was refluxed for 1 hour, treated with additional 1 N hydrochloric acid (2 mL) and refluxed for another 2 hours. The reaction solution was concentrated and the resulting residue partitioned between ethyl acetate and aqueous sodium bicarbonate solution. The organic layer was dried (sodium sulfate) and concentrated. The resulting pale yellow gum was purified by flash chromatography over silica gel (methylene chloride/2 N methanolic ammonia) to afford 1.40 g (92%) of Intermediate 2 as a colorless foamy solid: ^{1}H NMR ($CDCl_3$) δ 7.31–7.13 (m, 5H), 6.93–6.70 (m, 3H), 4.47 (d, J=8.5, 1H), 4.18 (s, 4H), 3.82 (t, J=5.9, 1H), 3.74 (d, J=6.0, 2H), 3.06 (d, J=8.5, 1H), 3.06–2.97 (m, 1H), 2.92–2.83 (m, 1H), 1.97–1.87 (m, 1H), 1.45–1.15 (m, 4H) ppm.

Intermediate 3

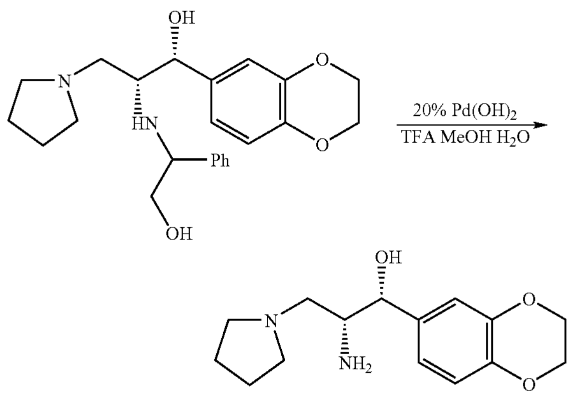
(1R,2R,1"S)-1-(2',3'-Dihydro-benzo[1,4]dioxin-6'-yl)-2-(2"-hydroxy-1"-phenyl-ethylamino)-3-pyrrolidin-1-yl-propan-1-o1



To a stirred solution of Intermediate 2 (1.38 g, 3.35 mmol) in tetrahydrofuran (30 mL) was added lithium aluminum hydride (0.26 g, 6.9 mmol). The foamy suspension was stirred overnight and then quenched with the addition (dropwise until frothing ceases) of 1 N aqueous sodium hydroxide (13 mL). The mixture was diluted with water and extracted with ethyl acetate. The organic layer was dried (sodium sulfate) and concentrated to afford a colorless gum. Flash chromatography over silica gel (methylene chloride/2 N methanolic ammonia) afforded 0.94 g (70%) of product as a colorless tacky foam: ^{1}H NMR ($CDCl_3$) δ 7.36–7.17 (m, 5H), 6.88–6.74 (m, 3H), 4.42 (d, J=5.4, 1H), 4.26 (s, 4H), 3.79–3.69 (m, 1H), 3.64–3.56 (m, 1H), 3.55–3.45 (m, 1H), 3.00–2.90 (m, 1H), 2.67–2.57 (m, 1H), 2.43–2.32 (m, 4H), 2.25–2.15 (m, 1H), 1.75–1.65 (m, 4H) ppm.

Intermediate 4

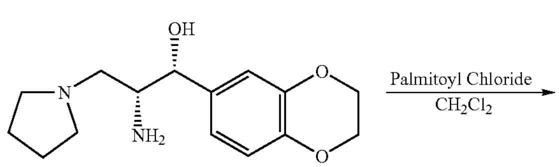
(1R,2R)-2-Amino-1-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-3-pyrrolidin-1-yl-propan-1-o1



In a high pressure reaction bomb equipped with a mechanical stirrer was loaded a solution of Intermediate 3 (0.91 g, 2.28 mmol) in 10:1 methanol/water (22 mL), trifluoroacetic acid (0.18 mL, 2.3 mmol) and 20% palladium hydroxide on carbon (Perlman's catalyst; 0.91 g). The reactor was evacuated and backfilled with argon three times and then evacuated and refilled with hydrogen (100 psi). The reaction was stirred for 2 days and then evacuated and flushed with nitrogen. The reaction solution was filtered through Celite and concentrated. The resulting gray-green gum was flash chromatographed over silica gel (methylene chloride/2 N methanolic ammonia) to afford 0.165 g (26%) of product as a near colorless gum: ^{1}H NMR ($CDCl_3$) δ 6.89–6.76 (m, 3H), 4.54 (d, J=3.7, 1H), 4.25 (s, 4H), 3.43 (s, 1H), 3.14–3.07 (m, 1H), 2.68–2.41 (m, 6H), 1.82–1.71 (m, 4H) ppm.

Compound 5

(1R,2R)-Hexadecanoic acid[2-(2',3'-dihydro-benzo [1,4]dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-ylm-ethyl-ethyl]-amide



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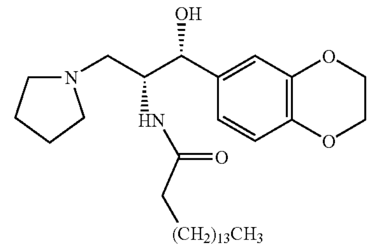
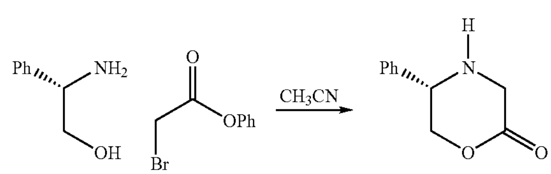


To a stirred solution of Intermediate 4 (0.165 g, 0.593 mmol) in methylene chloride (8 mL) was added palmitoyl chloride (0.18 g, 0.59 mmol) followed by N,N-diisopropylethylamine (0.11 mL, 0.65 mmol). The solution was stirred for 2 hours and then concentrated. The residue was partitioned between ethyl acetate and aqueous sodium bicarbonate solution. The organic layer was dried (sodium sulfate) and concentrated. The resulting off-white solid was flash chromatographed over silica gel (methylene chloride/2 N methanolic ammonia) to afford 0.174 g (57%) of product as a white solid. Comparisons by ^{1}H NMR spectroscopy and analytical chiral HPLC (column: Chirex (S)-VAL and (R)-NE, 4.6×250 mm; eluant: 0.5% trifluoroacetic acid in 67:31:2 hexane/methylene chloride/ethanol; flow: 1 mL/min; detection 280 nM) demonstrate that this material was identical to a sample of the same compound prepared by the method of Polt, et al. (*J. Org. Chem.,* 1998, 63, 8837). Enantiomeric excess was determined to be 99.6%. Total contamination from the two possible diastereomers is determined to be 0.2%. ^{1}H NMR ($CDCl_3$) δ 6.88–6.73 (m, 3H), 5.84 (d, J=7.3, 1H), 4.90 (d, J=3.8, 1H), 4.24 (s, 4H), 4.22–4.15 (m, 1H), 2.86–2.72 (m, 2H), 2.72–2.55 (m, 4H), 2.10 (t, J=7.5, 2H), 1.82–1.74 (m, 4H), 1.58–1.46 (m, 2H), 1.32–1.16 (m, 24H), 0.88 (t, J=6.7, 3H) ppm.

EXAMPLE 2

Large Scale Preparation of Ceramide-Like Compounds

(5S)-5-Phenylmorpholin-2-One

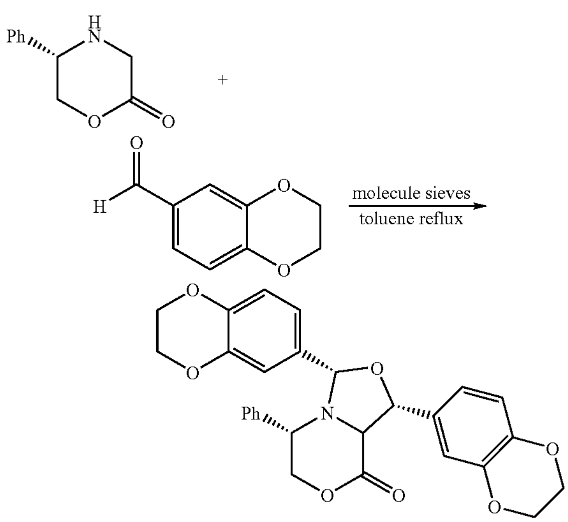


A solution of S-(+)-Phenyl glycinol (Aldrich, 10.17 g, 78.12 mmol) and Diisopropylethylamine (Aldrich, 34 mL, 195 mmol, 2.5 equivalents) was prepared in CH_3CN (200 mL). This solution was added to phenyl-α-bromoacetate (18.48 g, 85.9 mnol, 1.1 equivalents) dissolved in CH_3CN (50 mL) under nitrogen dropwise over 2 hours. The resulting solution was stirred under nitrogen for 16–20 hours. The solvent was removed by rotoevaporation keeping the bath temperature at below 25° C. To the oil was added ethyl acetate (120 mL) and the mixture was stirred for 15 minutes.

The resulting white precipitate was filtered off and the solid washed with ethyl acetate (25 mL). The filtrate was rotoevaporated to an oil keeping the bath temperature below 25° C. After drying under vacuum for 0.5 hours, the oil was dissolved in CH_2Cl_2 (17 mL) and loaded onto a silica gel column (60 g packed with 10% ethyl acetate/hexanes. The upper byproduct spots were eluted with 10% ethyl acetate/hexanes and the product was eluted with 50% ethyl acetate/hexanes-100% ethyl acetate. The fractions containing the product were rotoevaporated to an oil keeping the bath temperature below 25° C. This oil was dissolved in ethyl acetate (12 mL) and hexanes (60 mL) was added slowly in an ice bath to precipitate the product. The resulting precipitate was filtered. The white to yellow solid was vacuum dried. The (5S)-5-phenylmorpholin-2-one obtained (7.4 g, 41.8 mmol, 53%) was used directly in the next step.

Intermediate 1

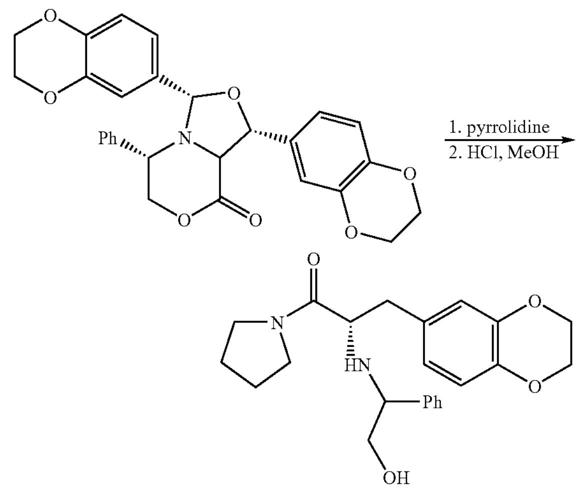
(1R,3S,5S,8aS)-1,3-Bis-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-5-phenyl-tetrahydro-oxazolo[4,3-c][1,4]oxazin-8-one



(5S)-5-Phenylmorpholin-2-one (7.4 g, 41.8 mmol) and benzodioxolane-6-carboxaldehyde (Aldrich or Alfa Aesar, 20.56 g, 125.2 mmol, 3.0 equivalents) was dissolved in toluene (180 mL). The solution was placed in a soxhlet extractor apparatus filled with 4 Å molecular sieves (ca 30 g). The solution was refluxed under nitrogen for 2–3 days. After cooling to room temperature, the solvent was removed by rotoevaporation and the oil was dissolved in ethyl acetate (200 mL). A solution of sodium bisulfite (Aldrich, 50 g) in water (100 mL) was added and the two phase mixture was stirred at room temperature for 1 hour. The resulting white solid was filtered off and washed with ethyl acetate. The filtrate was placed in a separatory funnel and the layers separated. The organic layer was washed with water (100 mL) and saturated sodium chloride solution (100 mL). The dried (Na_2SO_4) solution was filtered and rotoevaporated to a yellow-red foamy oil (23.11 g). After drying under vacuum for 1 hour, diethyl ether (350 ml) was added and the mixture was stirred at room temperature for 16–20 hours. The resulting white-yellow solid was filtered. The solid was dried under vacuum. The cycloadduct was obtained in 46% yield (9.34 g).

Intermediate 2

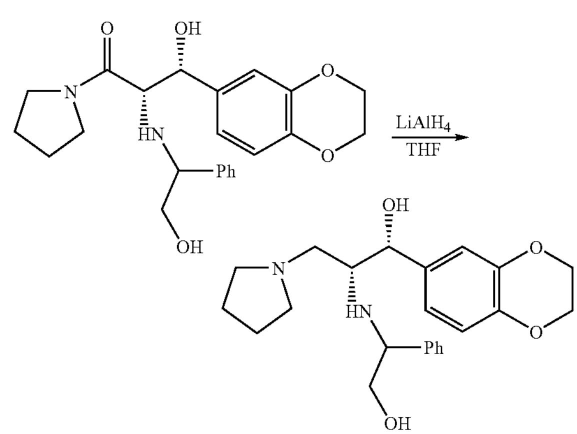
(2S,3R,1"S)-3-(2',3'-Dihydro-benzo[1,4]dioxin-6'-yl)-3-hydroxy-2-(2"-hydroxy-1"-phenyl-ethylamino)-1-pyrrolidin-1-yl-propan-1-one



To the cycloadduct (Intermediate 1, 6.7 g, 13.74 mmol) dissolved in methylene chloride (40 mL) was added pyrrolidine (Aldrich, 5.7 mL, 68.7 mmol, 5 equivalents). The solution was stirred under nitrogen at room temperature for 16–18 hours. The solvent was rotoevaporated to yield a yellow foamy oil which was vacuum dried for 0.5 hours. The crude was dissolved in methanol (115 mL) and a 1 M aqueous HCl solution (115 mL) was added. The solution was refluxed for 4 hours. After cooling to room temperature, the methanol was removed by rotoevaporation. Ethyl acetate (60 mL) was added and the two phase system was stirred at room temperature for 5–15 minutes. The two layers were separated and the organic layer was extracted with 1 M HCL (30 mL). The combined aqueous layers were washed two times with ethyl acetate (60, 30 mL). A saturated sodium bicarbonate solution (150 mL) was added to the aqueous layer slowly. The product was extracted three times with ethyl acetate (60 mL) from the basic (pH=8–9) aqueous layer. The combined organic layers containing the product were washed with a saturated sodium chloride solution (30 mL). After drying with Na_2SO_4 the solution was filtered and rotoevaporated to yield a yellow solid. Intermediate 2 was obtained in 93% yield (5.26 g).

Intermediate 3

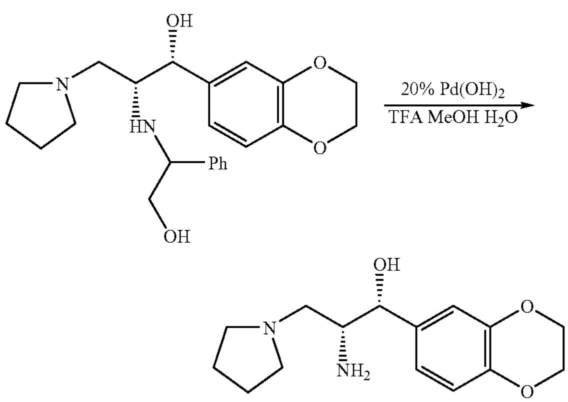
(1R,2R,1"S)-1-(2',3'-Dihydro-benzo[1,4]dioxin-6'-yl)-2-(2"-hydroxy -1'-phenyl-ethylamino)-3-pyrrolidin-1-yl-propan-1-ol



To a 3-neck flask equipped with a dropping funnel and condenser was added $LiAlH_4$ (Aldrich, 1.2 g, 31.7 mmol, 2.5 equivalents) and anhydrous THF (20 mL) under nitrogen. A solution of Intermediate 2 (5.23 g, 12.68 mmol) in anhydrous THF (75 mL) was added dropwise to the reaction over 15–30 minutes. The reaction was refluxed under nitrogen for 9 hours. The reaction was cooled in an ice bath and a 1M NaOH solution was carefully added dropwise. After stirring at room temperature for 15 minutes, water (50 mL) and ethyl acetate (75 mL) was added. The layers were separated and the aqueous layer was extracted twice with ethyl acetate (75 mL). The combined organic layers were washed with saturated sodium chloride solution (25 mL). After drying with Na_2SO_4 the solution was filtered and rotoevaporated to yield a colorless to yellow foamy oil. Intermediate 3 was obtained in 99% yield (5.3 g).

Intermediate 4

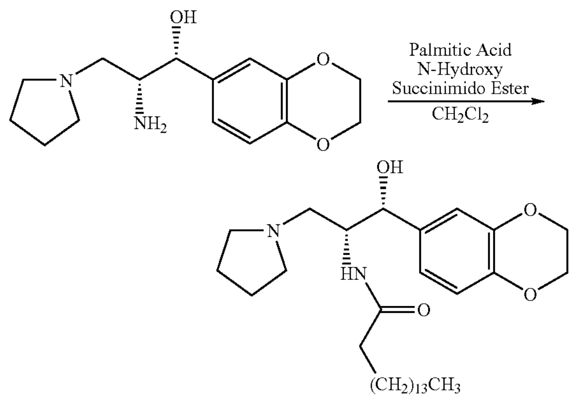
(1R,2R)-2-Amino-1-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-3-pyrrolidin-1-yl-propan-1-ol



Intermediate 3 (5.3 g, 13.3 mmol) was dissolved in methanol (60 mL). Water (6 mL) and trifluoroacetic acid (2.05 m/L, 26.6 mmol, 2 equivalents) were added. After being placed under nitrogen, 20% Palladium hydroxide on carbon (Pearlman's catalysis, Lancaster or Aldrich, 5.3 g) was added. The mixture was placed in a Parr Pressure Reactor Apparatus with glass insert. The apparatus was placed under nitrogen and then under hydrogen pressure 110–120 psi. The mixture was stirred for 2–3 days at room temperature under hydrogen pressure 100–120 psi. The reaction was placed under nitrogen and filtered through a pad of celite. The celite pad was washed with methanol (100 mL) and water (100 mL). The methanol was removed by rotoevaporation. The aqueous layer was washed with ethyl acetate three times (100, 50, 50 mL). A 10 M NaOH solution (10 mL) was added to the aqueous layer (pH=12–14). The product was extracted from the aqueous layer three times with methylene chloride (100, 100, 50 mL). The combined organic layers were dried with Na_2SO_4, filtered and rotoevaporated to a colorless oil. The foamy oil was vacuum dried for 2 h. Intermediate 4 was obtained in 90% yield (3.34 g).

Compound 5

(1R,2R)-Hexadecanoic acid[2-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-2-hydroxy -1-pyrrolidin-1-ylm-ethyl-ethyl]amide



To a solution of Intermediate 4 (3.34 g, 12.0 mmol) in methylene chloride (50 mL) was added a solution of palmitic acid N-hydroxylsuccinimide ester (Sigma, 4.24 g, 12.0 mmol) over 15–30 minutes under nitrogen at room temperature. The solution was stirred at room temperature for 18–20 hours. To the reaction was added methylene chloride (50 mL) and a 1 M NaOH solution (25 mL). The two phase system was stirred at room temperature for 15–30 min. Water (25 mL) was added and the layers were separated. The aqueous layer was back extracted with methylene chloride (25 mL). The combined organic layers were washed twice with water (25 mL) and once with a saturated sodium chloride solution (25 mL). The organic layer was dried with Na_2SO_4, filtered and rotoevaporated to a light yellow oil. The crude was recrystallized from hexane (50 mL). The white solid (5.46 g) obtained was separated on silica gel (300 g) with 2% methanol:methylene chloride-4% methanol:methylene chloride-4% 2 M ammonium in methanol: methylene chloride. The white solid obtained was recrystallized form hexanes (70 mL). Compound 5 was obtained in 66% yield (4.18 g). Analytical chiral HPLC (column: Chirex (S)-VAL and (R)-NE, 4.6×250 mm; eluant: 0.5% trifluoroacetic acid in 67:31:2 hexane/methylene chloride/ethanol; flow: 1 mL/min; detection: 280 nM) showed this material to be 98.98% pure with 0.89% of a diastereoisomer and 0.14% of the enantiomer.

EXAMPLE 3

Alternative Large Scale Preparation of Ceramide-Like Compounds

(5S)-5-Phenylmorpholin-2-one HCl salt

A solution of phenyl bromoacetate (Aldrich, 862.17 g, 4.0 moles, 1.1 equivalents) in acetonitrile (reagent grade, 1500 ml) was cooled in an ice bath (internal temperature below 5° C.). To this was added a cold slurry (internal temperature below 5° C.) of S-(+)-2-phenyl glycinol (Aldrich, 500 g, 3.65 moles, 1 equivalent) and diisopropylethylamine (DIPEA) (Aldrich, 1587 ml, 9.11 moles, 2.5 equivalents) in acetonitrile (2900 ml) in portions while keeping the internal temperature below 10° C. The mixture was stirred at this

temperature for 30 minutes before the ice bath was removed and the mixture was allowed to stir at room temperature for an additional 4 hours. The solvent was removed in vacuo while maintaining the bath temperature at 25° C. The mixture was coevaporated with ethyl acetate (2×500 ml) to produce a light yellow viscous oil. To the reaction mixture, ethyl acetate (4500 ml) was added and the flask was immersed in an ice bath with agitation. The mixture was allowed to cool below 8° C. The solid was filtered and washed with ethyl acetate (3×250 ml). The solution was cooled to below 5° C. Dry HCl gas was passed slowly into the solution while maintaining the internal temperature below 15° C. until the pH was below 2 (wet pH paper). The mixture was allowed to stir at this temperature and pH for an additional 20 minutes before the solid was suction filtered. The solid was washed with ethyl acetate (3×200 ml) and dried under high vacuum for about 20 hours. The yield was 412 g (53%). ^{1}H NMR was consistent with the (5S)-5-phenylmorpholin-2-one HCl salt.

Intermediate 1

(1R,3S,5S,8aS)-1,3-Bis-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-5-phenyl-tetrahydrooxazolo[4,3-c][1,4]oxazin-8-one

To a stirred suspension of (5S)-5-phenylmorpholin-2-one HCl salt (381 g, 1 equivalent) in 15% ethyl acetate in toluene (2270 ml) was added a solution of sodium bicarbonate (1.1 equivalents) in water (2000 ml). The resulting biphasic solution was stirred at room temperature for about 1 hour. The organic layer was transferred to a flask containing 1,4-benzodioxan-6-carboxaldehyde. The flask was then equipped with a Dean-Stark unit, a condenser and a nitrogen inlet. The mixture was heated at reflux with agitation while about 650 ml solvent (mixture of ethyl acetate and toluene) was collected via Dean-Stark unit. The resulting yellow-red solution was allowed reflux for about 64 hours, under nitrogen while the water formed during the reaction was collected in the Dean-Stark unit. Most of the solvent was then removed via distillation at atmospheric pressure through Dean-Stark unit. The residual solvent was then removed by coevaporation with heptane (500 ml) and tert-butylmethyl ether (2×725 ml) to produce a yellow semi solid product. The semi solid product was dissolved in ethyl acetate (3400 ml). A solution of sodium bisulfite (920 g) in water (1500 ml) was added and the mixture was allowed to stir at room temperature for about 1 hour. The solid that was formed was removed by filtration and washed with ethyl acetate (3×400 ml). The filtrate was washed with water (1450 ml), 5% brine solution (1450 ml) and dried over $MgSO_4$ (100 g). The solvent was removed in vacuo to afford a yellow solid. To this was added tert-butylmethyl ether (2900 ml) and the suspension was stirred at room temperature for 20 to 22 hours. The yellow solid was suction filtered, washed with tert-butylmethyl ether (2×600 ml) and dried under high vacuum at room temperature for about 22 hours. The yield was 400.5 g (58%). ^{1}H NMR and TLC were consistent with Intermediate 1.

Intermediate 2

2S,3R,1"S)-3-(2',3'-Dihydro-benzo[1,4]dioxin-6'-yl)-3-hydroxy-2-(2"hydroxy-1"-phenyl-ethylamino)-1-pyrrolidin-1-yl-propan-1-one

A solution of Intermediate 1 (312 g, 0.64 moles), pyrrolidine (267 ml, 3.2 moles, 5 equivalents) and tetrahydrofuran (1350 ml) was heated at reflux for 4.5 hours under nitrogen atmosphere. The solvent and excess pyrrolidine were removed in vacuo to produce the crude intermediate as an orange viscous oil. The oil was dissolved in methanol (3000 ml) and 1M hydrochloric acid solution (3000 ml). The resulting solution was heated at reflux for about 7 hours. The solvent was then removed in vacuo to afford a mixture of an oil and water. To this ethyl acetate (2000 ml) was added and the aqueous layer was separated. The organic layer was extracted with aqueous 1M HCl (1000 ml). The aqueous layers were combined and washed with ethyl acetate (2000 ml). The aqueous layer was cooled in an ice bath. The pH of the aqueous layer was adjusted to about 9 (pH paper) with 10 M aqueous NaOH (525 ml). The aqueous layer was extracted with ethyl acetate (3000 ml). The organic layer was washed with 5% brine solution (1000 ml) and dried (Na_2SO_4). The solvent was removed in vacuo to produce a yellow viscous oil. The yield was 213.4 g, 81%. ^{1}H NMR was consistent with Intermediate 2.

Intermediate 3

1R,2R,1"S)-1-(2',3'-Dihydro-benzo[1,4]dioxin-6'-yl)-2-(2"-hydroxy-1"-phenyl-ethylamino)-3-pyrrolidin-1-yl-propan-1-ol

To a slurry of $LiAlH_4$ (50.7 g, 1.34 moles, 2.6 equivalents) in tetrahydrofuran (700 ml) was added a solution of Intermediate 2 (213.34 g, 0.517 moles) in tetrahydrofuran (2000 ml) slowly with agitation at room temperature. The mixture was refluxed for about 4 hours. TLC analysis (10% methanol in methylene chloride, v/v) indicated consumption of the starting material. The reaction mixture was cooled in an ice bath (below 5° C.) and water (135 ml) was added very slowly while keeping the internal temperature less than or equal to 10° C. To this was then added a 15% aqueous NaOH solution (70 ml) followed by water (200 ml). The reaction mixture was allowed to warm to room temperature while the agitation was continued. Methylene chloride (1000 ml) was then added to the mixture and the salts were filtered through a pad of celite. The salts were washed with methylene chloride (2×500 ml). The filtrates were combined and the solvent was removed in vacuo to produce a yellow oil. The oil was dissolved in 1M aqueous HCl (1500 ml) and washed with ethyl acetate (3×500 ml). The aqueous layer was cooled in an ice bath to below 5° C. and the pH of the aqueous layer was adjusted to 12 to 13 with a 10 M aqueous NaOH solution (220 ml) keeping the internal temperature at less than or equal to 10° C. The mixture was allowed to warm to room temperature. The aqueous layer was extracted with methylene chloride (2×500 ml). The organic layers were combined and washed with brine solution (500 ml), dried (Na_2SO_4) and the solvent was removed in vacuo to afford a yellow viscous oil. The yield was 186.4 g (88.5%). ^{1}H NMR was consistent with Intermediate 3.

Intermediate 4 Dioxalate Salt

(1R,2R)-2-Amino-1-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-3-pyrrolidin-1-1-yl-propan-1-ol dioxalate salt

A suspension of Intermediate 3 (358 g, 0.90 moles), ethanol (1500 ml), 1M HCl solution (1500 ml) and 10% $Pd(OH)_2$ (32 g, 20 weight %) were hydrogenated at about 50 psi for about 36 h at room temperature. The mixture was filtered through a Cuono filter. The Cuono filter was washed with 10% ethanol in water (500 ml). The filtrates were

combined and ethanol was removed in vacuo. The aqueous layer was extracted with ethyl acetate (3×600 ml). The organic layer was extracted with 1M HCl aqueous (700 ml). The aqueous layers were combined and cooled in an ice bath (0 about 5° C.). The pH of the aqueous layer was adjusted to about 12 (pH paper) with 10 M aqueous NaOH solution (490 ml) keeping the internal temperature below 10° C. The aqueous layer was allowed to warm to room temperature. The aqueous layer was extracted with methylene chloride (2×1500 ml, 1×750 ml). The combined organic layers were dried over $MgSO_4$ and the solvent was removed in vacuo to afford a yellow viscous oil. The crude weight was 214.3 g (86%). ^{1}H NMR was consistent with Intermediate 4.

A solution of oxalic acid (152.4 g, 1.693 moles, 2.2 equivalents) in methylisobutyl ketone (2300 ml) was added slowly with stirring to a solution of Intermediate 4 (214.3 g, 0.77 moles, 1 equivalent) in methylisobutyl ketone (800 ml) at room temperature. The resulting mixture was stirred at room temperature for about 2.5 hours. The solid was filtered, and triturated with acetone (2000 ml) at room temperature for about 16 hours. The solid was filtered, washed with acetone (3×100 ml) and dried under high vacuum to produce an off-white solid. The yield was 312.5 g (89%). ^{1}H NMR was consistent with Intermediate 4 dioxalate salt.

Compound 5

(1R,2R)-Hexadecanoic acid[2-(2',3'-dihydro-benzo [1,4]dioxin-6'-yl)-2-hydroxy -1-pyrrolidin-1-ylm-ethyl-ethyl]-amide

To a cold solution (about 5° C.) of Intermediate 4 dioxalate salt (507 g, 1.11 moles) in water (10 L) was added a 10 M aqueous NaOH solution (500 ml) with stirring while keeping the internal temperature below 10° C. The solution was allowed to warm to room temperature while the pH of the solution was maintained at about 14 (pH paper). The aqueous layer was extracted with methylene chloride (3×6000 ml). The organic layers were combined, washed with water (2000 ml), dried ($MgSO_4$) and the solvent was removed in vacuo to afford a yellow viscous oil, Intermediate 4. The yield was 302 g (98%). ^{1}H NMR was consistent with Intermediate 4.

A solution of palmitic acid NHS-ester (Sigma, 382.5 g, 1.01 equivalents) in methylene chloride (2500 ml) was added to a solution of intermediate 4 (302 g) in methylene chloride (1500 ml) at room temperature over a period of 1.25 hours under a nitrogen atmosphere. The mixture was allowed to stir at room temperature for about 18 hours. A solution of 1M aqueous NaOH (2425 ml) was added and the mixture was stirred at room temperature for about 3 hours. The organic layer was separated and the aqueous layer was extracted with methylene chloride (800 ml). The organic layers were combined, washed with a 1M NaOH solution (3×1500 ml) and water (1500 ml). The organic layer was dried over $MgSO_4$ and the solvent was removed in vacuo to afford a semi solid. The semi-solid was coevaporated with heptane (3×100 ml). The crude product was transferred to a 12 L three-necked RB flask and heptane (7500 ml) was added. The mixture was heated at reflux with stirring under a nitrogen atmosphere. The solution was slowly cooled to about 55° C. (internal temperature) and poured into another flask. The solution was stirred at room temperature for 24 hours under a nitrogen atmosphere. The off white solid was filtered, washed with heptane (2×500 ml) and dried under high vacuum for 24 hours. The solid (397 g) was transferred to a 12 L RB flask and 30% ethyl acetate in heptane (8000 ml) was added. The resulting mixture was heated at reflux for 30 minutes with stirring. The solution was cooled to about 55° C. (internal temperature) and poured into another flask. The stirring was continued at room temperature under a nitrogen atmosphere for about 24 hours. The solid was filtered, washed with heptane (2×100 ml) and dried under high vacuum to afford an off white solid. The yield was 324 g (58%). ^{1}H NMR and TLC were consistent with Compound 5. mp 96.1° C. HPLC analysis: chiral purity 99.7%, chemical purity 99.7%. Anal. Calcd for $C_{31}H_{52}N_2O_4$: C, 72.05; H, 10.14; N, 5.42. Found C, 72.03; H, 10.19; N, 5.42.

EXAMPLE 4

Preparation of Compounds 6–8

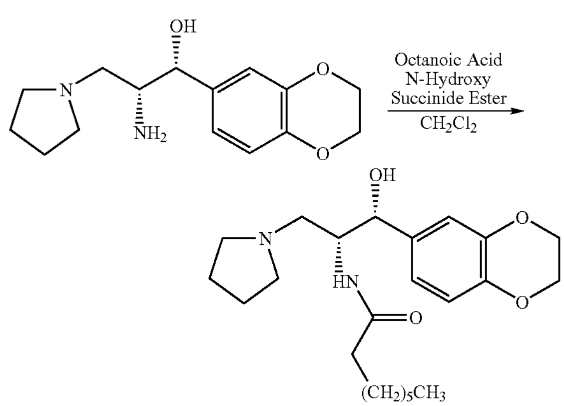
N-hydroxysuccinimide esters of fatty acids were prepared by the method of Lapidot, Y. Rappoport, S. and Wolman, Y. *Journal of Lipid Research* 8, 1967 or as described below:

Octanoic Acid N-Hydroxysuccinimide Ester

N-hydroxysuccinimide (Aldrich, 20.0 g, 173 mmol) and triethyl amine (29 mL, 208 mmol) were dissolved in methylene chloride in an ice bath under nitrogen. Octanoyl chloride (Aldrich, 35 mL, 205 mmol) was added dropwise over 0.5 hours. The ice bath was removed and the solution with a white solid was stirred for 1 hour at room temperature. The white solid was removed by filtration and the filtrate was washed with water (100 mL) and saturated aqueous sodium bicarbonate (100 mL). The organic layer was dried with sodium sulfate, filtered and heptane (100 mL) was added. The solution was rotoevaporated to remove most of the methylene chloride and leave a colorless to white flaky precipitate in heptane. The precipitate was filtered and washed with heptane. After drying, Octanoic acid N-hydroxysuccinimide ester was obtained in 84% yield (35.4 g).: ^{1}H NMR ($CDCl_3$) 2.84 (br s, 4H), 2.60 (t, J=7.48 Hz, 2H), 1.78–1.71 (m, 2H), 1.42–1.26 (m, 8H), 0.88 (t, J=6.7 Hz, 3H) ppm.

Compound 6

(1R,2R)-Octanoic acid[2-(2',3'-dihydro-benzo[1,4] dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide

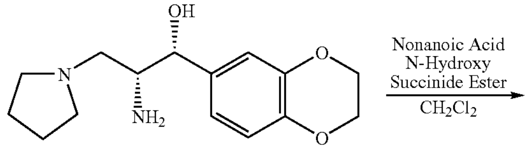


Compound 6

To Intermediate 5 (22.36 g, 80.33 mmol) dissolved in anhydrous methylene chloride (300 mL) was added a solution of octanoic acid N-hydroxysuccinimide ester (19.4 g, 80.39 mmol) dissolved in anhydrous methylene chloride (150 mL) over 15–30 minutes under nitrogen at room temperature. The solution was stirred at room temperature for 18–20 hours. To the reaction was added 1M aqueous NaOH solution (200 mL). The two phase system was stirred at room temperature for 45 minutes. The layers were separated and the combined organic layers were washed twice with 1 M NaOH (2×200 mL) and twice with water (2×100 mL). The organic layer was dried with sodium sulfate, filtered and rotoevaporated to a yellow oil. Most of the crude material was dissolved in 5% ethyl acetate in heptane (1 L) at reflux. After cooling to 40° C., the hazy solution was separated from the yellow oil by decanting the solution into a new flask. The first flask was rinsed twice with 5% ethyl acetate in heptane (2×250 mL) by the same process (reflux and cooling to 40° C. and decanting the solution from the oil). The combined solution was heated to reflux and allowed to cool to room temperature over 4 hours. The resulting white solid was filtered and washed with 5% ethyl acetate in heptane (100 mL) and heptane (100 mL). The white solid (13.9 g) was dried under vacuum for 16–24 hours. This solid was mostly dissolved in 5% ethyl acetate in heptane (800 mL) at reflux. After cooling to 50° C., the hazy solution was separated from the yellow oil by decanting the solution into a new flask. The first flask was rinsed with 5% ethyl acetate in heptane (100 mL) by the same process (reflux and cooling to 50° C. and decanting the solution from the oil). The combined solution was heated to reflux and allowed to cool to room temperature over 4 hours. The resulting white solid was filtered and washed with 5% ethyl acetate/heptane (50 mL) and heptane (50 mL). After drying at room temperature under vacuum for 2–3 days, Compound 6 was obtained in 39% yield (12.57 g). Analytical chiral HPLC (column: Chirex (S)-VAL and (R)-NE, 4.6×250 mm) showed this material to be 99.9% the desired R,R isomer. Analytical HPLC showed this material to be 99.6% pure. mp 87–88° C. ^{1}H NMR ($CDCl_3$) δ 6.86–6.73 (m, 3H), 5.84 (d, J=7.3 Hz, 1H), 4.91 (d, J=3.4 Hz, 1 H), 4.25 (s, 4H), 4.24–4.18 (m, 1H), 2.85–2.75 (m, 2H), 2.69–2.62 (m, 4H), 2.10 (t, J=7.3 Hz, 2 H), 1.55–1.45 (m,2 H), 1.70–1.85 (m, 4H), 1.30–1.15 (m, 8H), 0.87 (t, J=6.9 Hz, 3H) ppm.

Compound 7

(1R,2R)-Nonanoic acid[2-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide



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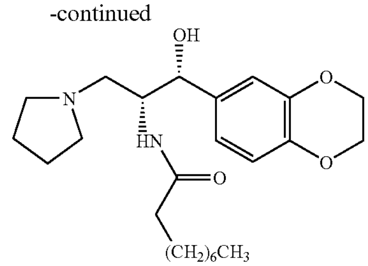
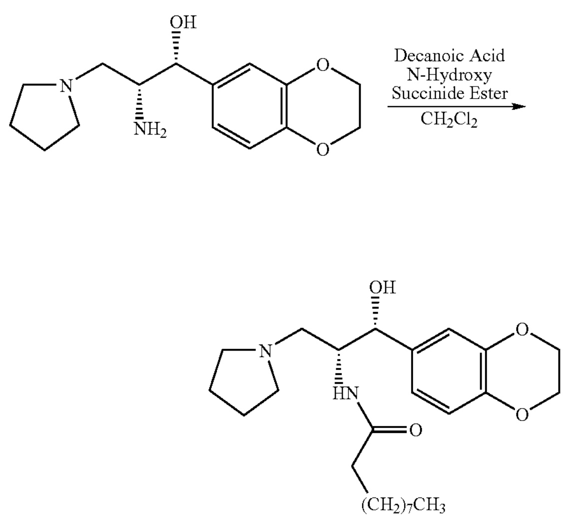


Compound 7

This compound was prepared by the method described for Compound 6 using Nonanoic acid N-hydroxysuccinimide ester. Analytical HPLC showed this material to be 98.4% pure. mp 74–75° C. ^{1}H NMR ($CDCl_3$) δ 6.86–6.76 (m, 3H), 5.83 (d, J=7.3 Hz, 1H), 4.90 (d, J=3.3 Hz, 1H), 4.24 (s, 4H), 4.24–4.18 (m, 1H), 2.85–2.75 (m, 2H), 2.69–2.62 (m, 4H), 2.10 (t, J=7.3 Hz, 2H), 1.55–1.45 (m, 2H), 1.70–1.85 (m, 4H), 1.30–1.15 (m, 10H), 0.87 (t, J=6.9 Hz, 3H) ppm.

Compound 8

(1R,2R)-Decanoic[2-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide



Compound 8

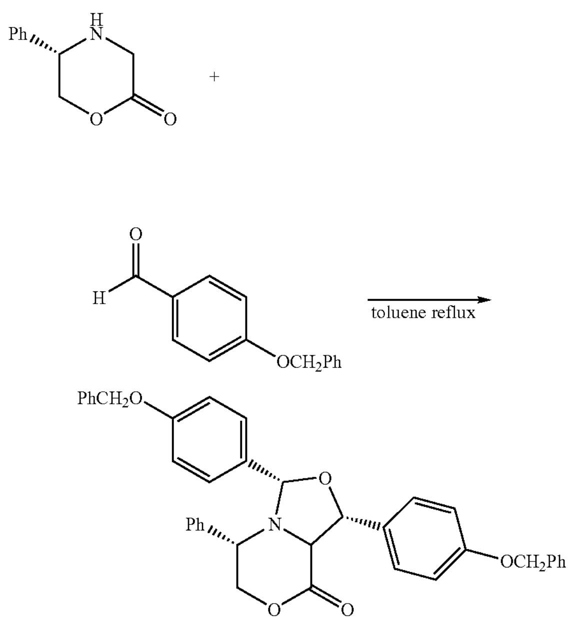
This compound was prepared by the method described for Compound 6 using decanoic acid N-hydroxysuccinimide ester. Analytical HPLC showed this material to be 99.3% pure. mp 97.5–98.5° C. ^{1}H NMR ($CDCl_3$) δ 6.86–6.76 (m, 3H), 5.83 (d, J=7.5 Hz, 1H), 4.90 (d, J=3.4 Hz, 1H), 4.24 (s, 4H), 4.24–4.18 (m, 1H), 2.85–2.75 (m, 2H), 2.69–2.62 (m, 4H), 2.10 (t, J=7.5 Hz, 2H), 1.55–1.45 (m, 2 H), 1.70–1.85 (m, 4H), 1.30–1.15 (m, 12H), 0.87 (t, J=6.8 Hz, 3H) ppm.

EXAMPLE 5

Preparation of Compound 13

Intermediate 9

(1R,3S,5S,8aS)-1,3-Bis-(4-benzyloxy-phenyl)-5-phenyl-tetrahydro-oxazolo[4,3-c][1,4]oxazin-8-one

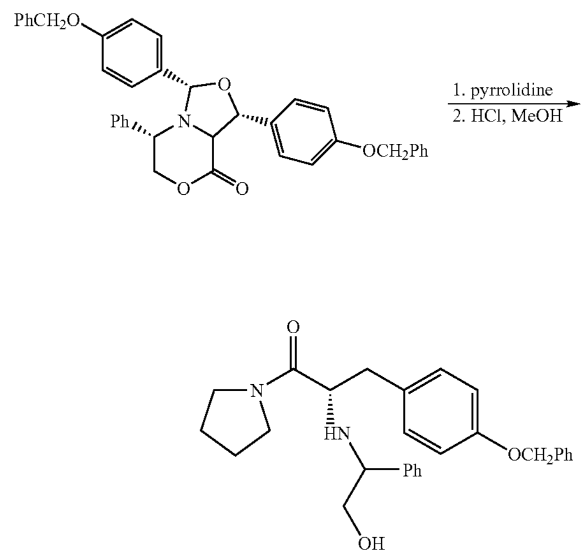


The (5S)-5-phenylmorpholin-2-one.HCl salt (57.45, 268.9 mmol) was stirred with ethyl acetate (500 mL) and saturated aqueous sodium bicarbonate (250 mL) for 30 minutes, until the biphasic solution was clear. The phases were separated, and the aqueous layer was extracted with ethyl acetate (2×250 mL). The combined organic phases were washed with saturated sodium chloride solution (250 mL). The organic layer was dried with sodium sulfate, filtered, concentrated to an oil, and dried under vacuum for 60 minutes. The 5-(S)-phenyl morpholin-2-one was obtained in a 86% yield (40.98 g, 231.3 mmol).

The 5-(S)-phenyl morpholin-2-one (40.98 g, 231.3 mmol) and 4-benzyloxybenzaldehyde (Aldrich, 147.3 g, 694 mmol, 3.0 equivalents) was dissolved in toluene (750 mL). The reaction was fitted with a Dean Stark Trap and a reflux condenser. The solution was refluxed under nitrogen for 2 days. After cooling to room temperature, the solvent was removed by rotoevaporation and the oil was dissolved in ethyl acetate (500 mL). A solution of sodium bisulfite (Aldrich, 125 g) dissolved in water (250 mL) was added and the two phase mixture was stirred at room temperature for 3 hours. The resulting white solid was filtered off and washed with ethyl acetate. The filtrate was placed in a separatory funnel and the layers separated. The organic layer was washed with water (250 mL), saturated aqueous sodium chloride solution (250 mL) and then dried (sodium sulfate) filtered and rotoevaporated to a foamy oil (144 g). After drying under vacuum for 1 hour, tert-butyl methyl ether (1450 mL) was added and the mixture was stirred at room temperature for 5 hours. The resulting white-yellow solid was filtered. The solid was dried under vacuum. Intermediate 9 was obtained in 27% yield (41.64 g, 71.46 mmol). ^{1}H NMR ($CDCl_3$) δ 7.5–6.8 (m, 23H), 5.0 and 5.1 (2 s, 4H), 4.5–4.3 (m, 2H), 4.2–4.1 (m, 2H) ppm.

Intermediate 10

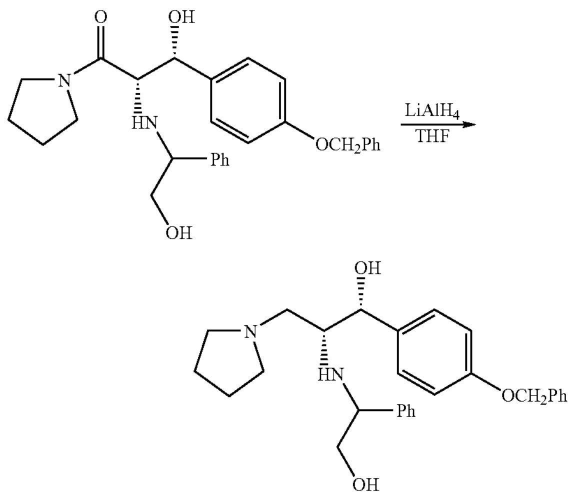
(2S,3R,1"S)-3-(4-Benzyloxy-phenyl)-3-hydroxy-2-(2"-hydroxy-1"-phenyl -ethylamino)-1-pyrrolidin-1-yl-propan-1-one



To Intermediate 9 (45.1 g, 77.4 mmol) dissolved in tetrahydrofuran (250 mL) was added pyrrolidine (Aldrich 33 mL, 395 mmol, 5.1 equivalents). The solution was stirred capped under nitrogen at room temperature for 16–18 hours. The solvent was rotoevaporated to yield a yellow foamy oil which was vacuum dried for 0.5 hours. The crude was dissolved in methanol (220 mL) and a 1M aqueous HCl solution (220 mL) was added. The solution was refluxed for 4 hours. After cooling to room temperature, the methanol was removed by rotoevaporation. To the resulting oil was slowly added 10 M aqueous NaOH (22 mL to adjust the pH to 14). The product was extracted three times with methylene chloride (300,100,100 mL) from the basic aqueous layer. After drying with sodium sulfate the combined organic layer was filtered and rotoevaporated to yield a yellow-orange foamy solid. Tert-butyl methyl ether (300 mL) was added and the mixture was stirred at room temperature for 7 hours. The resulting white-yellow solid was filtered, washed with tert-butyl methyl ether (50 mL) and vacuum dried. Intermediate 10 was obtained in 83% yield (29.77 g). ^{1}H NMR ($CDCl_3$) δ 7.4–7.2 (m, 12H), 6.9–6.8 (m, 2H), 5.05 (AB quartet, 2H), 4.47 (d, J=8.5, 1H), 3.9–3.3 (m, 3H), 3.05 (d, J=8.5, 1H), 3.0–2.8 (m, 2H), 2.3–2.2 (m, 1H), 1.85–1.7 (m, 1H), 1.45–1.15 (m, 4H) ppm.

Intermediate 11

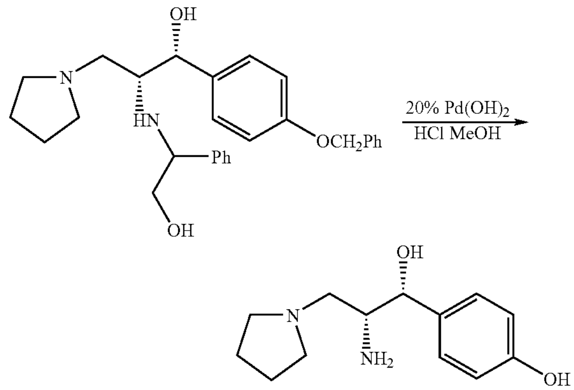
(1R,2R,1"S)-1-(4-Benzyloxy-phenyl)-2-(2"-hydroxy-1"-phenyl-ethylamino)-3-pyrrolidin-1-yl-propan-1-ol



In a 3-neck flask with dropping funnel and condenser under nitrogen was added $LiAlH_4$ (Aldrich, 6.3 g, 166 mmol, 2.57 equivalents) and anhydrous tetrahydrofuran (75 mL). A solution of Intermediate 10 (29.7 g, 64.48 mmol) in anhydrous tetrahydrofuran (300 mL) was added dropwise to the reaction over 15–30 minutes. The reaction was refluxed under nitrogen for 9 hours. The reaction was cooled in an ice bath and water (7.0 mL) was very carefully added drop by drop (vigorous exothermic reaction with hydrogen being given off). A 15% aqueous NaOH solution (7.0 mL) was added dropwise followed by water (21 mL). Halfway through the final water addition a large amount of a white solid formed. It was broken up by the addition of methylene chloride (250 mL). After stirring at room temperature for 15 minutes, the mixture was filtered through a celite plug (17 cm in diameter by 1 cm in height). The precipitate was washed with methylene chloride (2×250 mL). The filtrate was rotoevaporated to an oil. The oil was dissolved in 1M aqueous HCl (300 mL). This aqueous layer was washed with tert-butyl methyl ether (2×200 mL). After cooling in an ice bath, 10 M aqueous NaOH (35 mL) was carefully added to the aqueous layer (final pH=14). The product was extracted three times with methylene chloride (300 mL, 200 mL and 100 mL). After drying with sodium sulfate, the solution was filtered and rotoevaporated to yield a white solid. After drying, the Intermediate 11 was obtained in 94% yield (26.9 g). ^{1}H NMR ($CDCl_3$) δ 7.46–7.115 (m, 12H), 6.98–6.96 (m, 2H), 5.08 (s, 2H), 4.49 (d, J=4.7, 1H), 3.70–3.65 (m, 1H), 3.60–3.55 (m, 1H), 3.54–3.45 (m, 1H), 3.00–2.90 (m, 1H), 2.7–2.6 (m, 1H), 2.36 (br s, 4H), 2.15–2.05 (m, 1H), 1.70 (br s, 4H) ppm.

Intermediate 12

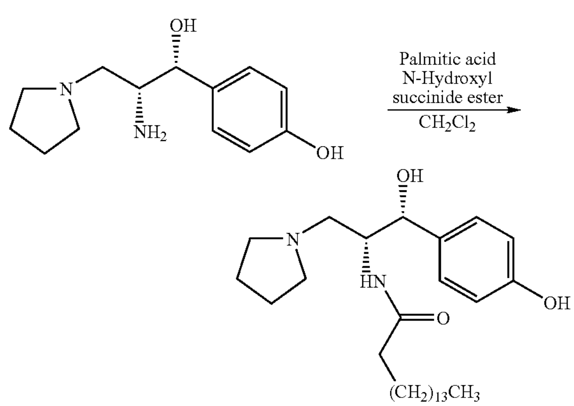
(1R,2R)-2-Amino-1-(4-benzyloxy-phenyl)-3-pyrrolidin-1-yl-propan-1-ol Hydrogen chloride salt



Intermediate 11 (26.9 g, 60.24 mmol) was dissolved in methanol (400 mL) and 1M aqueous HCl (130 mL) was added. After being placed under nitrogen, 20% palladium hydroxide on carbon (Pearlman's catalysis, Aldrich, 10.8 g) was added. The reaction was placed under nitrogen and then under hydrogen by evacuation and filling to a balloon. The mixture was stirred for 48 hours at room temperature under a hydrogen balloon. The reaction was placed under nitrogen and filtered through a pad of celite. The celite pad was washed with 10% water in methanol (250 mL) and water (50 mL). The solvent was removed by rotoevaporation and coevaporation with toluene (3×100 mL). The foamy solid was dissolved in isopropanol (300 mL) at reflux. The solution was cooled to room temperature and tert-butyl methyl ether (550 mL) was added. After stirring at room temperature for 2 hours, the white solid was filtered and washed with tert-butyl methyl ether. After drying, Intermediate 12 was obtained in ca 99% yield (18 g). ^{1}H NMR (DMSO-d6) δ 9.68 (br s, 1H), 8.53 (br s, 2H) 7.24 (d, J=8.55 Hz, 2H) 6.80 (d, J=8.55 Hz, 2H), 4.72 (d, J=7.0 Hz, 1H), 3.8–3.6 (m, 2H), 3.4–3.6 (m, 3H), 3.0–3.2 (m,2H), 2.7–2.5 (br s, 1H), 2.0–1.7 (br s, 4H) ppm.

Compound 13

(1R,2R)-Hexadecanoic acid[2-(4-benzyloxy-phenyl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide

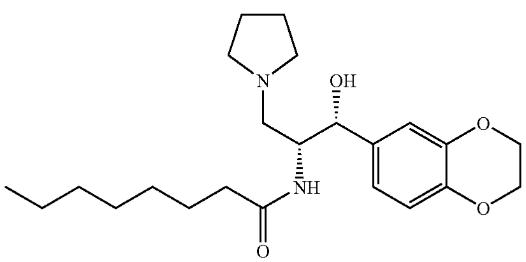


To Intermediate 12 (16.17 g 49.36 mmol) suspended in tetrahydrofuran (500 mL) was added triethylamine (28 mL, 4 equivalents). A solution of Palmitic acid N-hydroxysuccinimide ester (Sigma, 19.2 g, 54.29 mmol) dissolved in tetrahydrofuran (125 mL) was added over 30 minutes under nitrogen at room temperature. The solution was stirred at room temperature for 18–20 hours. The white precipitate was removed by filtration and the filtrate was rotoevaporated to a foamy off-white solid (35.5 g). The crude material was dissolved in methylene chloride (500 mL) and washed with water (100 mL) and saturated aqueous sodium carbonate solution (100 mL). After drying with sodium sulfate, the solution was filtered and rotoevaporated to yield a off-white foamy solid (24.75 g). This material was recrystallized from 40% ethyl acetate in heptane (500 mL, hot filtration). Compound 13 was obtained in 61% yield (14.45 g) Analytical chiral HPLC showed this material to be 99.7% the desired R,R isomer. Analytical HPLC showed this material to be 99.6% pure. mp 95–97° C. ^{1}H NMR ($CDCl_3$) δ 7.15 (d, J=8.5 Hz, 2H), 6.70 (d, J=8.5 Hz, 2H), 6.0 (d, J=7.3, 1H), 4.96 (d, J=3.8, 1H), 4.3–4.2 (m, 1H), 2.9–2.7 (m, 2H), 2.65–2.55 (m, 4H), 2.10 (t, J=7.5, 2H), 1.75 (br s, 4H), 1.58–1.46 (m, 2H), 1.32–1.16 (m, 24H), 0.9 (t, J=6.7, 3H) ppm.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

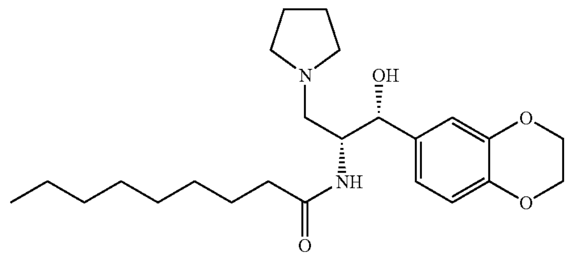
What is claimed is:

1. A compound represented by the following structural formula:



or a physiologically acceptable salt thereof.

2. A compound represented by the following structural formula:



or a physiologically acceptable salt thereof.

3. The compound of claim **1**, wherein the compound has an enantiomeric excess of at least 10%.

4. The compound of claim **1**, wherein the compound has an enantiomeric excess of at least 25%.

5. The compound of claim **1**, wherein the compound has an enantiomeric excess of at least 50%.

6. The compound of claim **1**, wherein the compound has an enantiomeric excess of at least 75%.

7. The compound of claim **1**, wherein the compound has an enantiomeric excess of at least 90%.

8. The compound of claim **1**, wherein the compound has an enantiomeric excess of at least 95%.

9. The compound of claim **1**, wherein the compound has an enantiomeric excess of at least 99%.

10. The compound of claim **2**, wherein the compound has an enantiomeric excess of at least 10%.

11. The compound of claim **2**, wherein the compound has an enantiomeric excess of at least 25%.

12. The compound of claim **2**, wherein the compound has an enantiomeric excess of at least 50%.

13. The compound of claim **2**, wherein the compound has an enantiomeric excess of at least 75%.

14. The compound of claim **2**, wherein the compound has an enantiomeric excess of at least 90%.

15. The compound of claim **2**, wherein the compound has an enantiomeric excess of at least 95%.

16. The compound of claim **2**, wherein the compound has an enantiomeric excess of at least 99%.

* * * * *

EXHIBIT C

US007253185B2

(12) **United States Patent**
Shayman et al.

(10) **Patent No.: US 7,253,185 B2**
(45) **Date of Patent: *Aug. 7, 2007**

(54) **AMINO CERAMIDE-LIKE COMPOUNDS AND THERAPEUTIC METHODS OF USE**

(75) Inventors: **James A. Shayman**, Ann Arbor, MI (US); **David J. Harris**, Lexington, MA (US); **Craig Siegel**, Woburn, MA (US); **Carol A. Nelson**, Westford, MA (US); **Diane P. Copeland**, North Billerica, MA (US)

(73) Assignees: **The Regents of the University of Michigan**, Ann Arbor, MI (US); **Genzyme Corporation**, Cambridge, MA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 72 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **11/119,541**

(22) Filed: **Apr. 29, 2005**

(65) **Prior Publication Data**

US 2005/0267094 A1 Dec. 1, 2005

Related U.S. Application Data

(63) Continuation of application No. 10/839,497, filed on May 5, 2004, now Pat. No. 6,916,802, which is a continuation of application No. 10/134,315, filed on Apr. 29, 2002, now abandoned.

(51) **Int. Cl.**
A61K 31/445 (2006.01)
A61K 31/40 (2006.01)
C07D 207/04 (2006.01)
C07D 211/06 (2006.01)

(52) **U.S. Cl.** **514/315**; 546/184; 546/246; 548/566; 548/567; 548/950; 514/408; 514/428

(58) **Field of Classification Search** 546/184, 546/246; 548/566, 567, 950; 514/408, 428, 514/315
See application file for complete search history.

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Primary Examiner—Golman M. M. Shameem
(74) *Attorney, Agent, or Firm*—Hamilton, Brook, Smith & Reynolds, P.C.

(57) **ABSTRACT**

The present invention provides amino ceramide-like compounds which inhibit glucosyl ceramide (GlyCer) formation by inhibiting the enzyme GlyCer synthase, thereby lowering the level of glycosphingolipids. The compounds of the present invention have improved GlcCer synthase inhibition activity and are therefore useful in therapeutic methods for treating various conditions and diseases associated with altered glycosphingolipid levels.

4 Claims, 12 Drawing Sheets

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Radin, N. S., et al., "Use of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Prophanol (PDMP), an Inhibitor of Glucosylceramide Synthesis," In NeuroProtocols, A Companion to Methods in Neurosciences, S. K. Fisher et al., Ed., (Academic Press, San Diego) 3:145-155 (1993).
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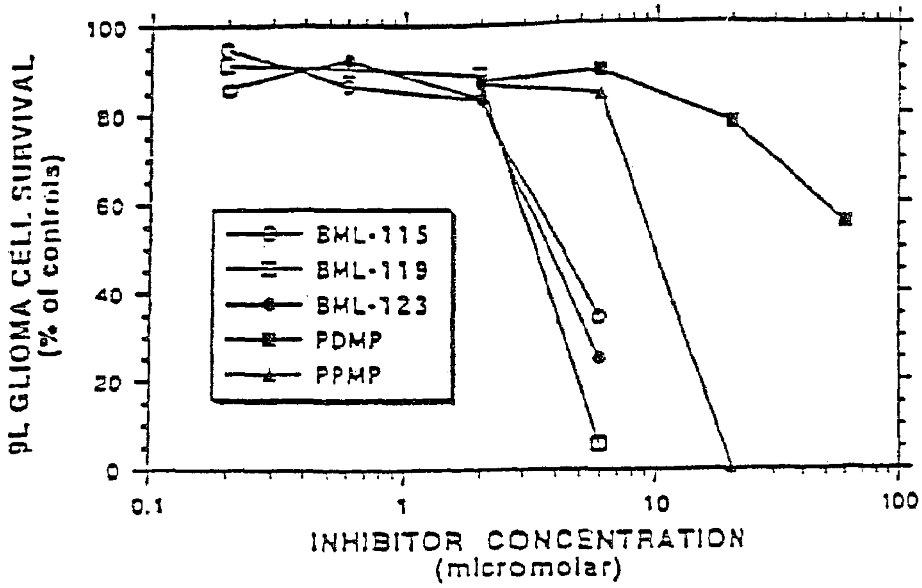
Figure 1



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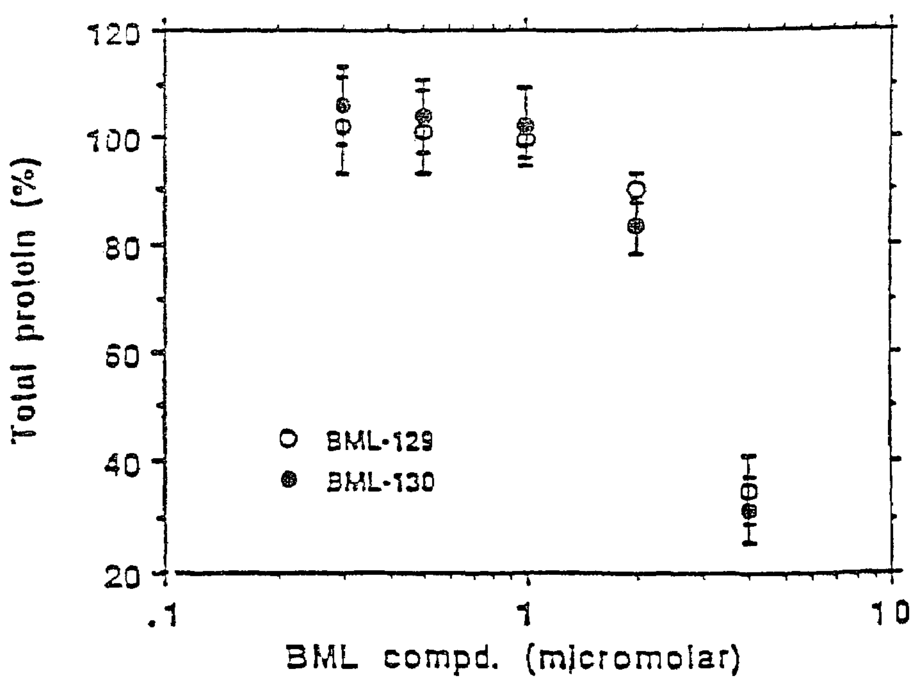

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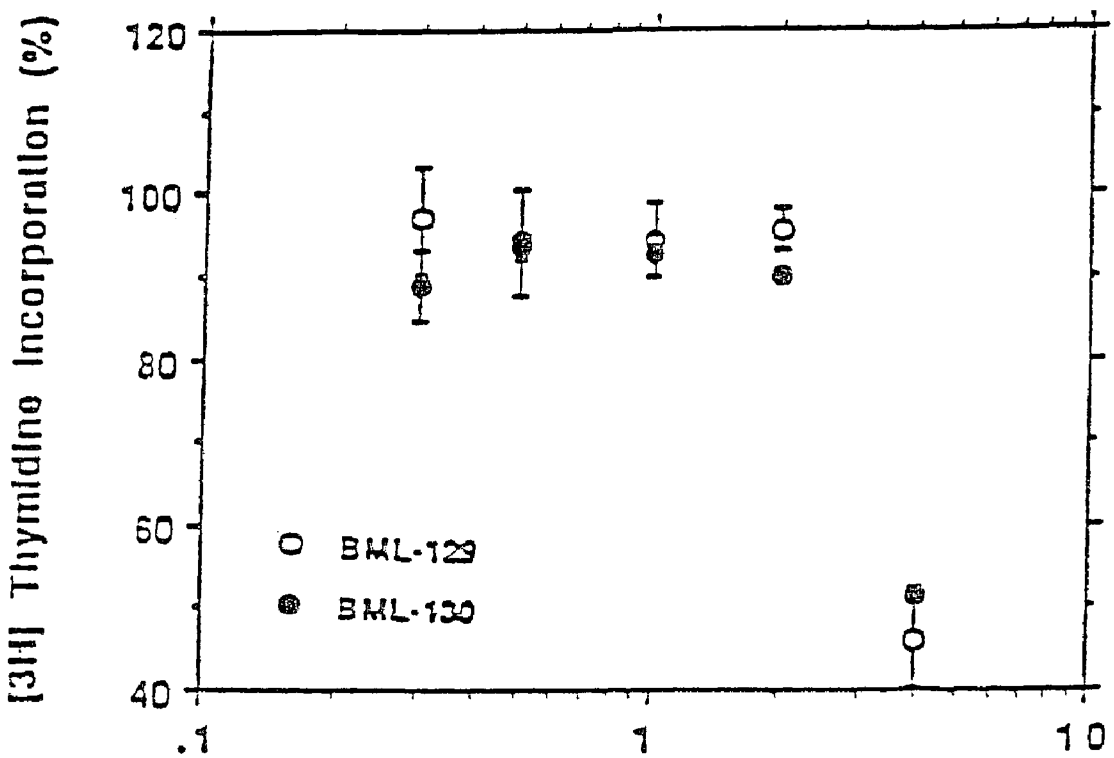
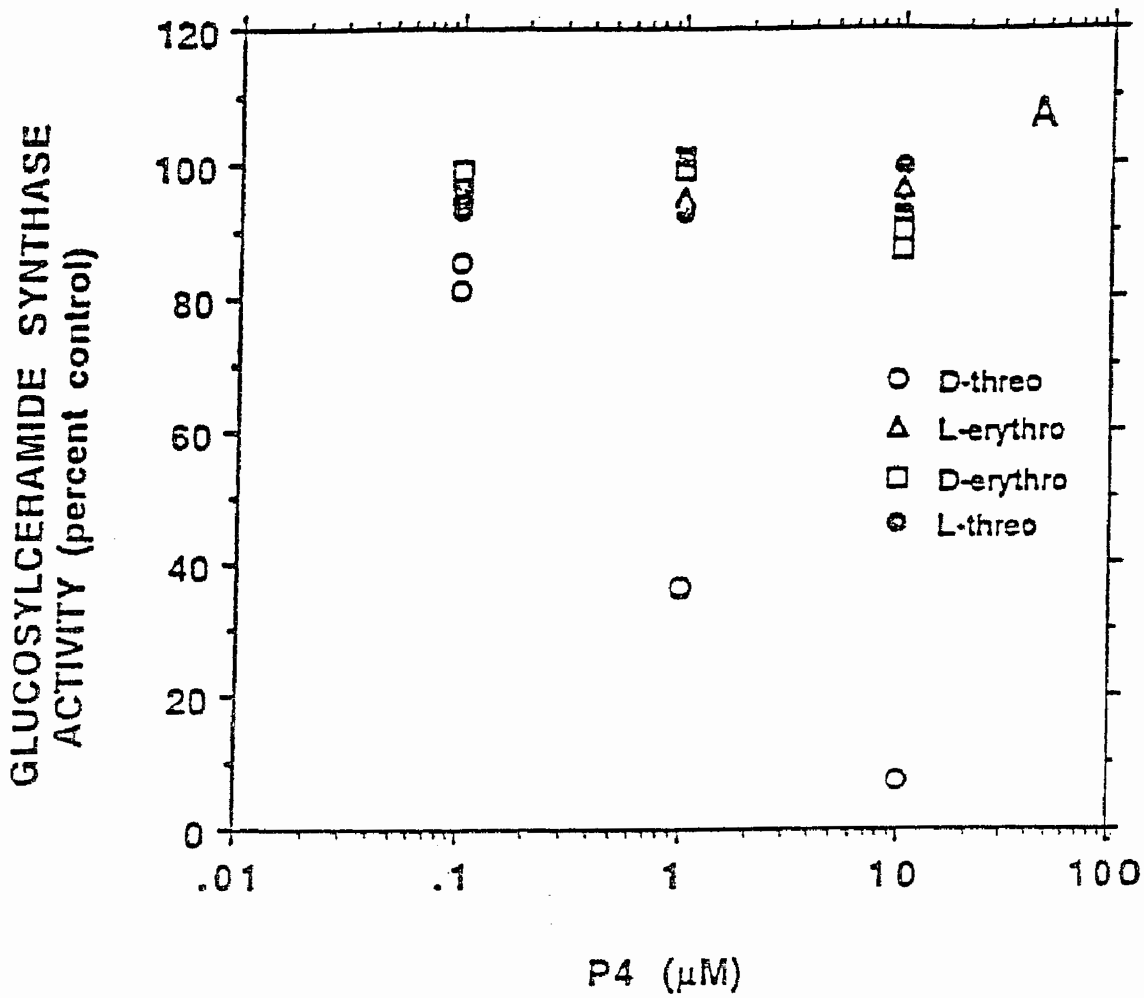


[3H] Thymidine Incorporation (%)
120
100
80
60
40
.1
1
10
BML-129
BML-130



120
100
80
60
40
20
0
GLUCOSYLCERAMIDE SYNTHASE ACTIVITY (percent control)
A
D-threo
L-erythro
D-erythro
L-threo
.01
.1
1
10
100
P4 (μM)

Figure 4A

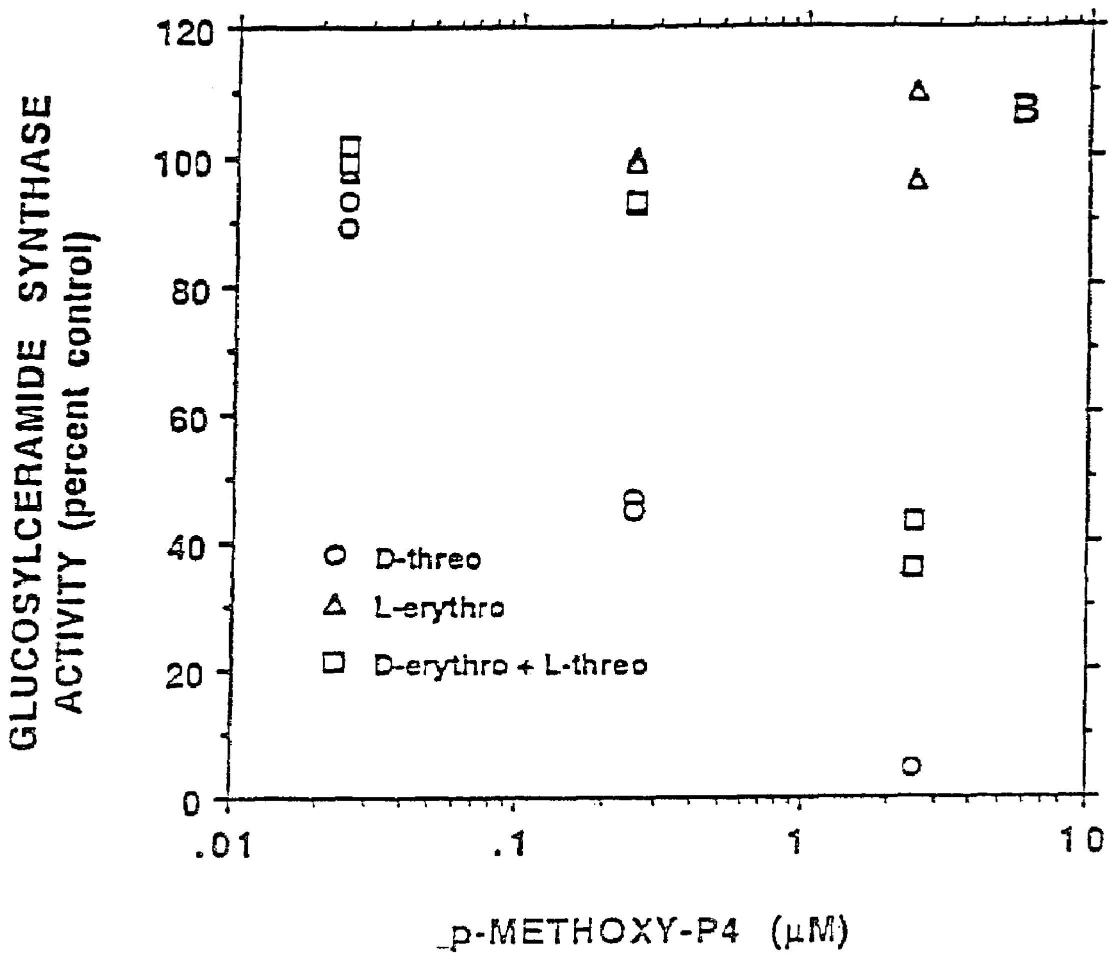
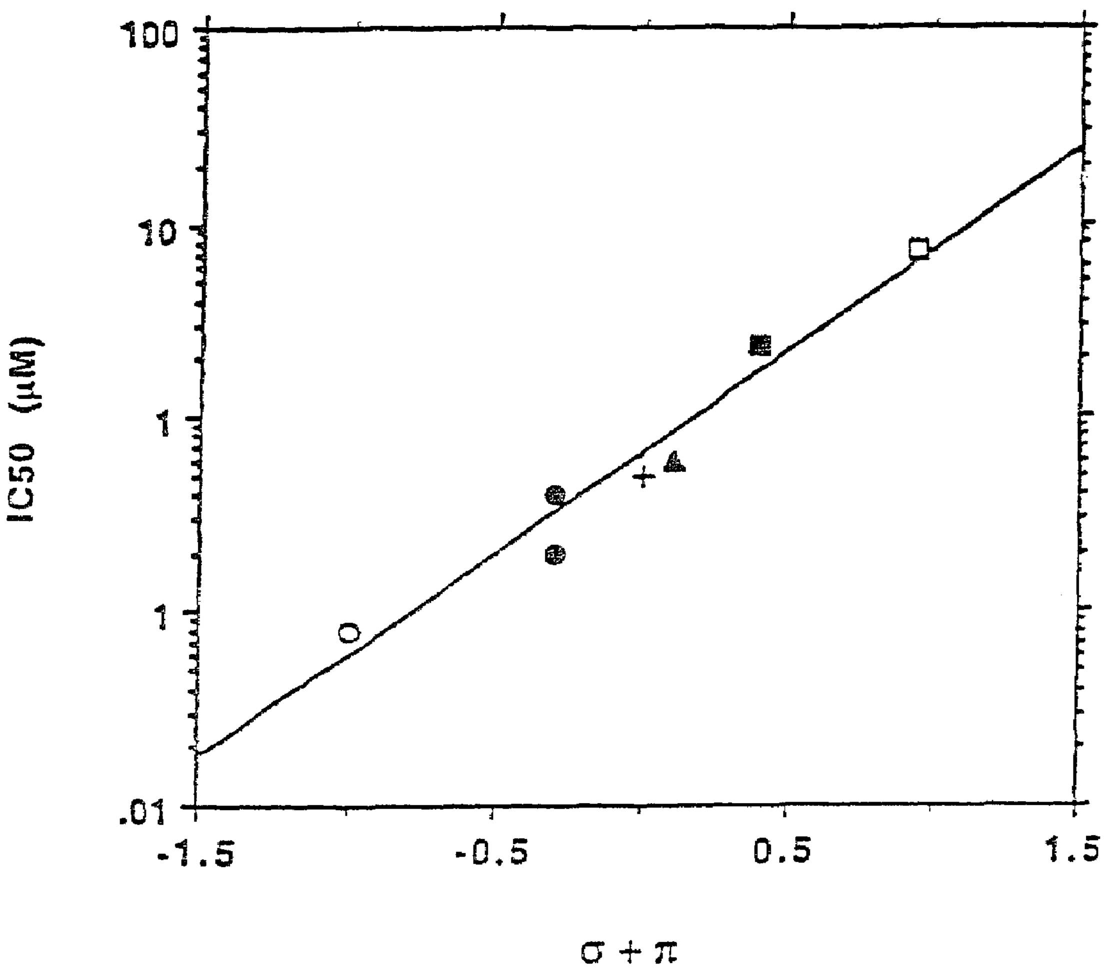

GLUCOSYLCERAMIDE SYNTHASE ACTIVITY (percent control)
120
100
80
60
40
20
0
.01
.1
1
10
B
D-threo
L-erythro
D-erythro + L-threo
p-METHOXY-P4 (μM)

Figure 4B



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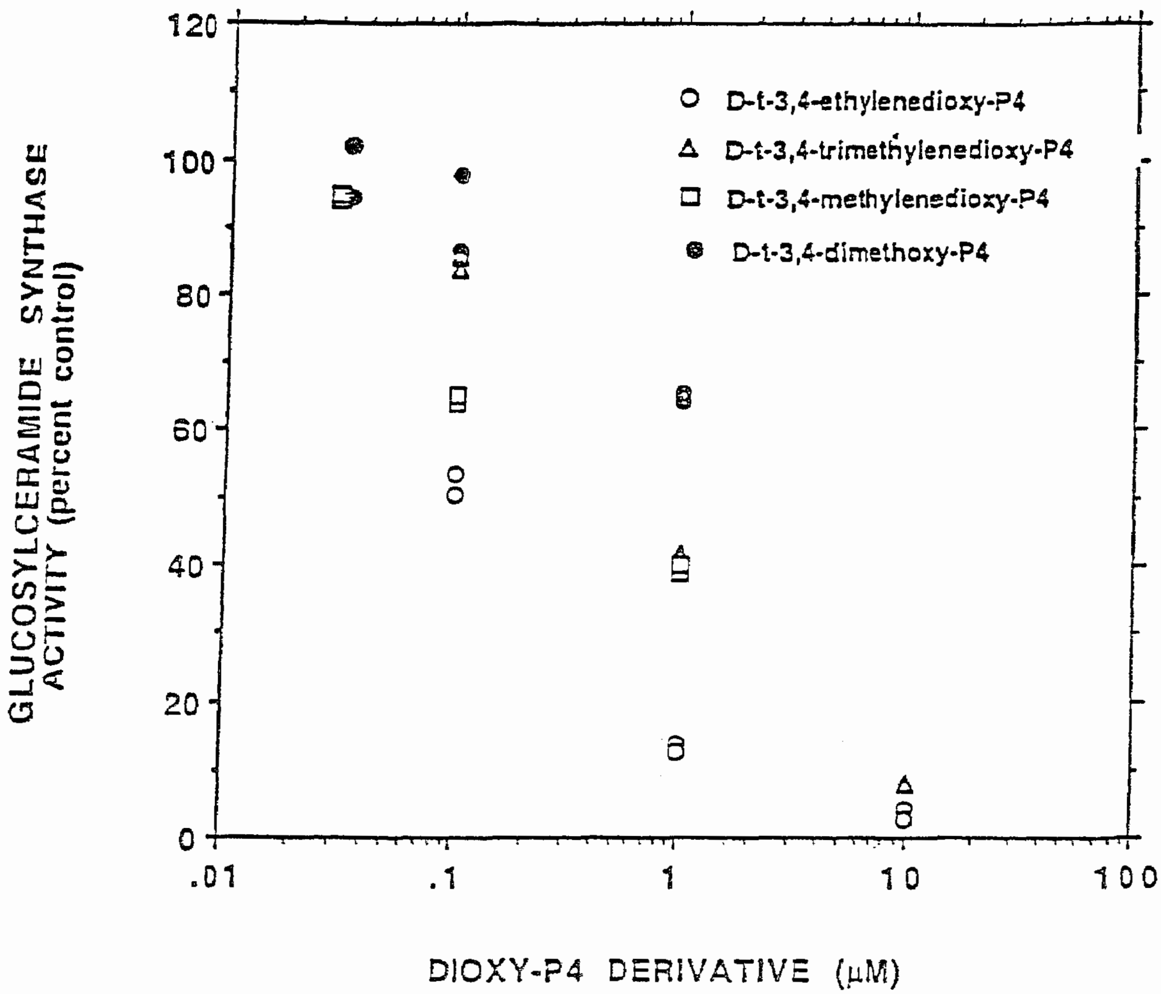


GLUCOSYLCERAMIDE SYNTHASE ACTIVITY (percent control)
120
100
80
60
40
20
0
.01
.1
1
10
100
DIOXY-P4 DERIVATIVE (μM)
D-t-3,4-ethylenedioxy-P4
D-t-3,4-trimethylenedioxy-P4
D-t-3,4-methylenedioxy-P4
D-t-3,4-dimethoxy-P4

Figure 6

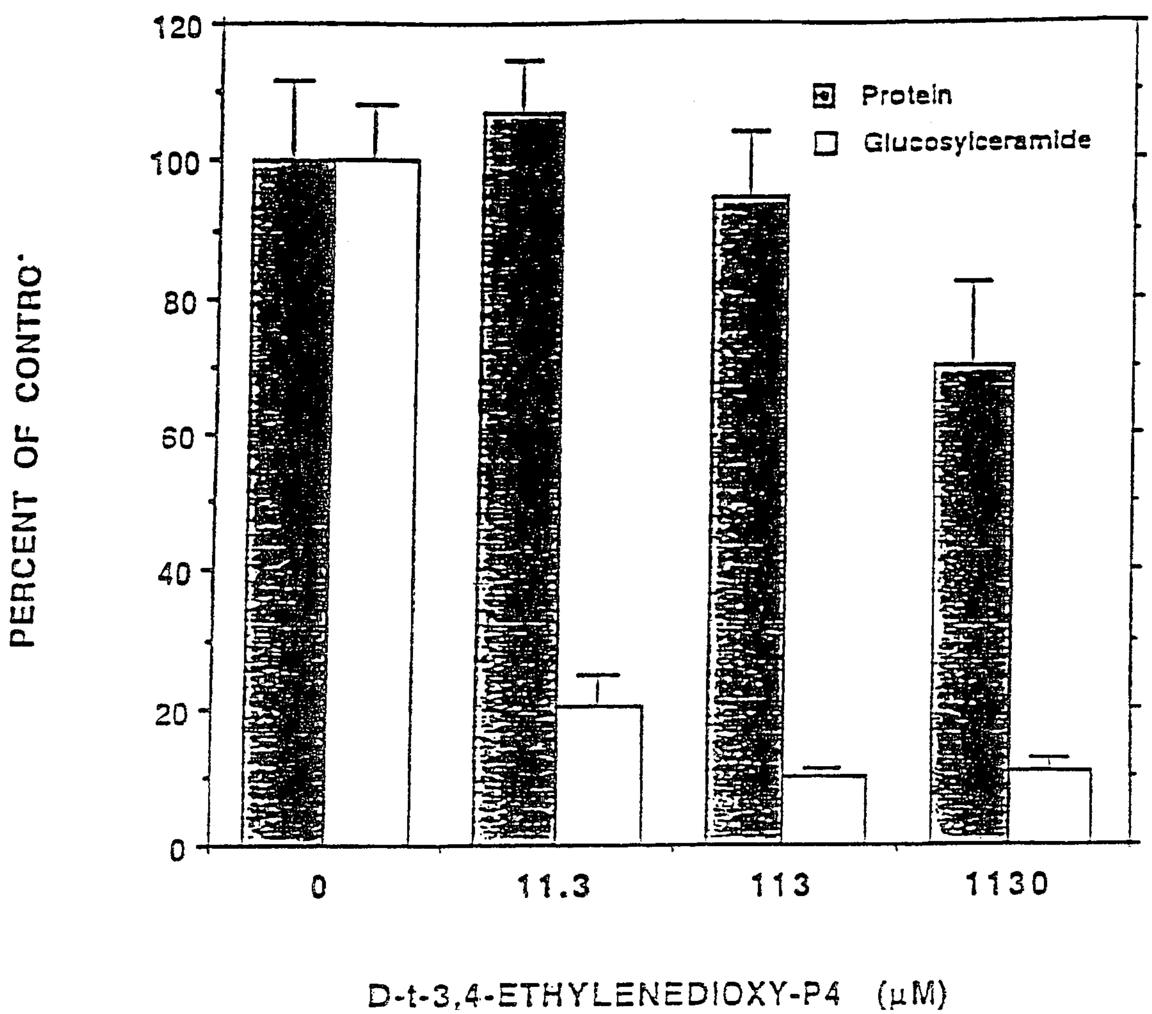

120
100
80
60
40
20
0
PERCENT OF CONTRO'
Protein
Glucosylceramide
0
11.3
113
1130
D-t-3,4-ETHYLENEDIOXY-P4 (μM)

Figure 7

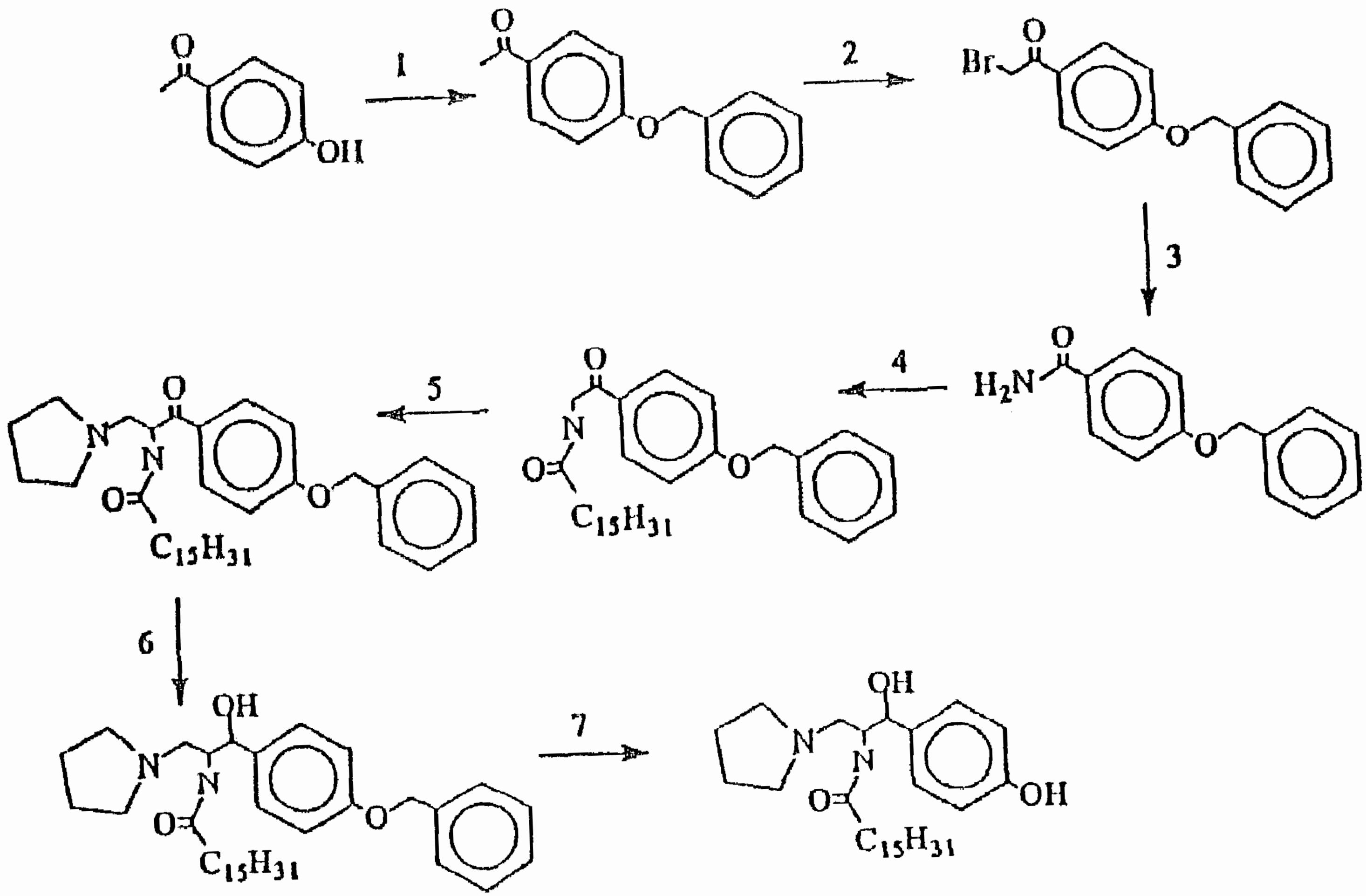
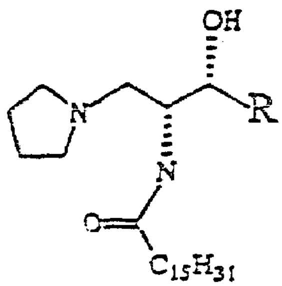
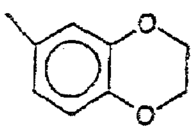


Figure 8

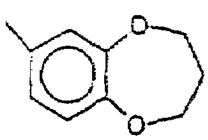


R=

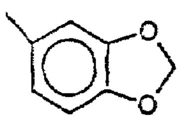
D-*threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4)



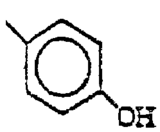
D-*threo*-1-(3',4'-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol



D-*threo*-1-(3',4'-trimethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol



D-*threo*-1-(3',4'-methylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol



D-*threo*-4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4)

Figure 9

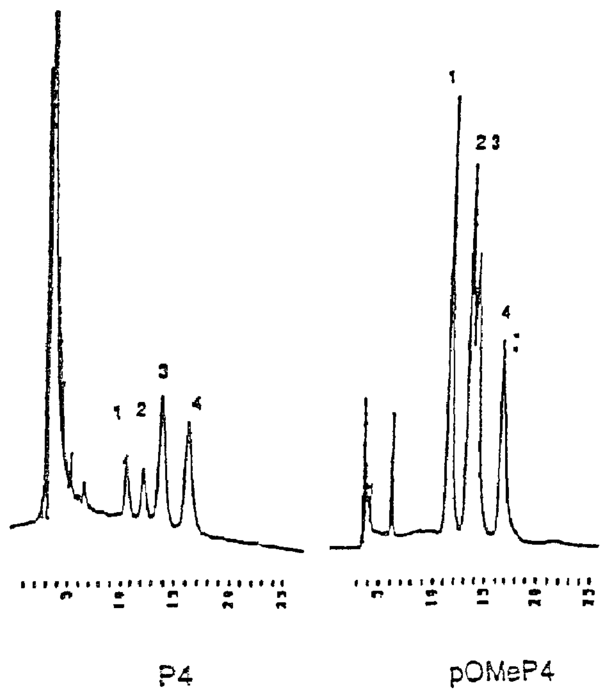


Figure 10

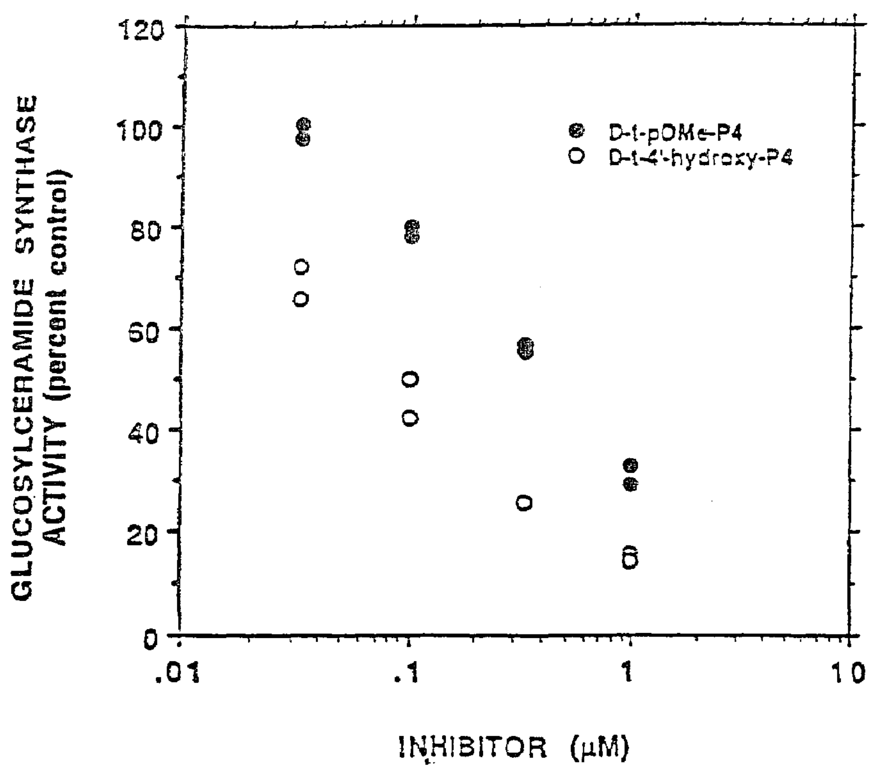


Figure 11

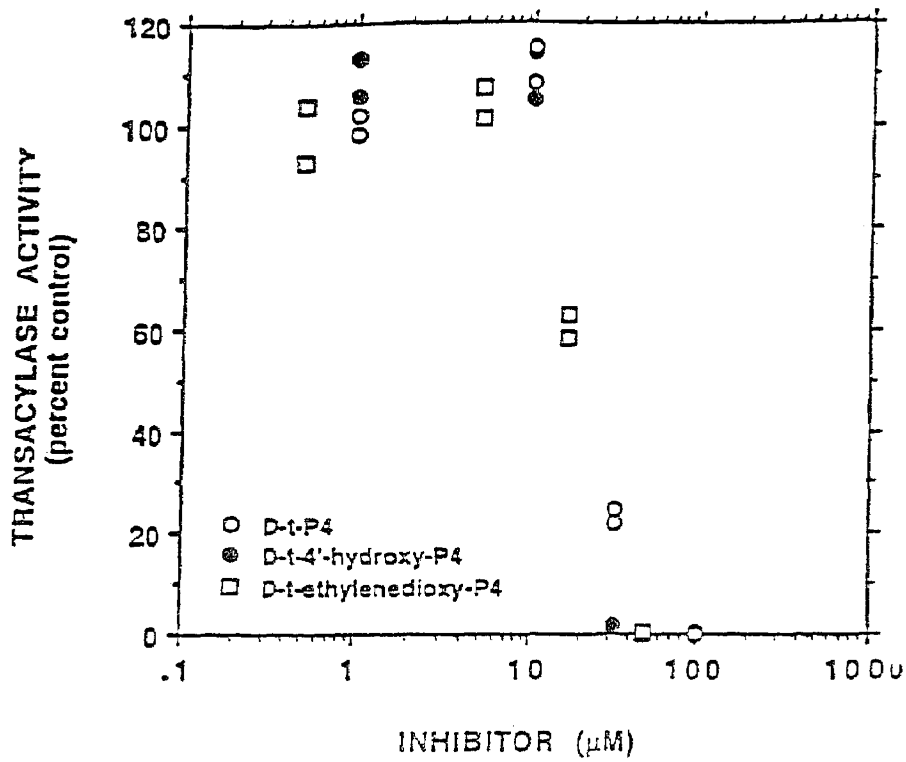


Figure 12

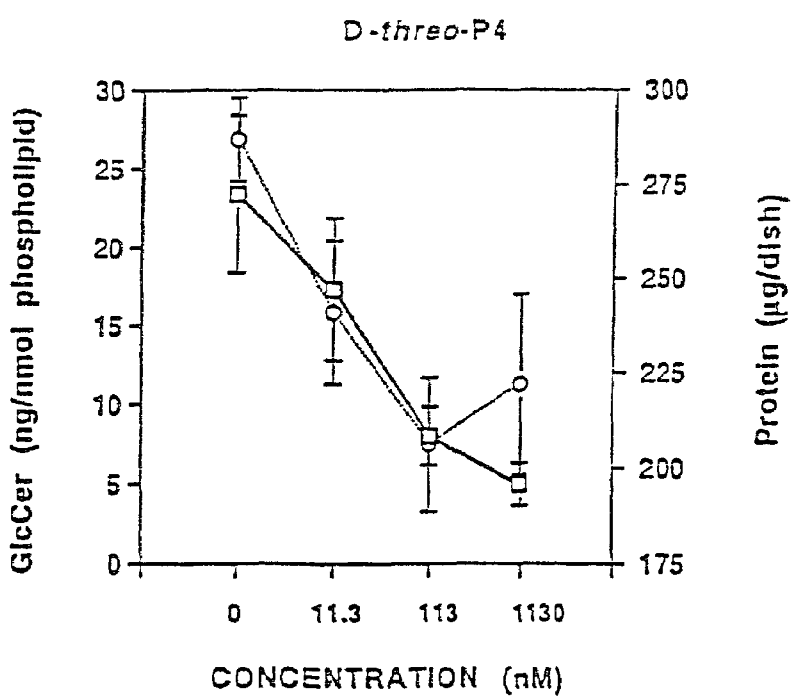


Figure 13

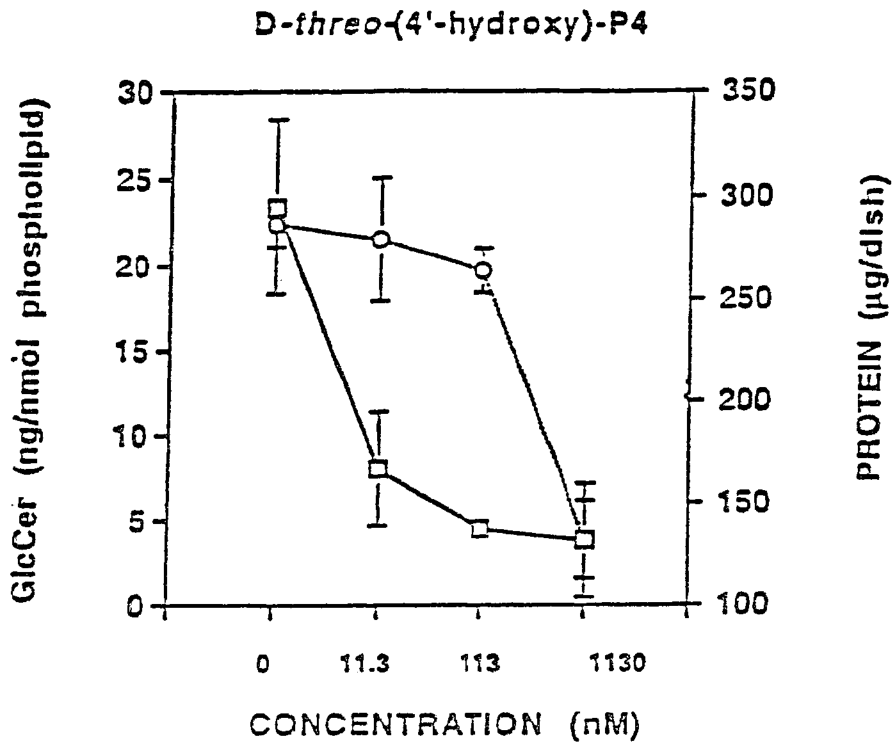


Figure 14

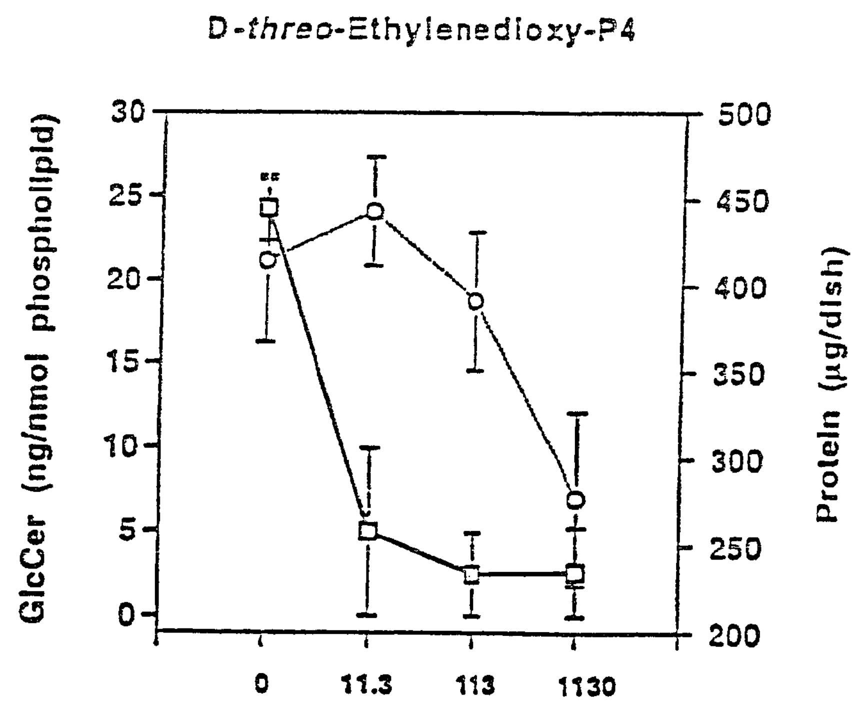


Figure 15

AMINO CERAMIDE-LIKE COMPOUNDS AND THERAPEUTIC METHODS OF USE

RELATED APPLICATIONS

This application is a continuation of U.S. Application Ser. No. 10/839,497, filed May 5, 2004 now U.S. Pat. No. 6,916,802, which is a continuation of U.S. application Ser. No. 10/134,315, filed Apr. 29, 2002, now abandoned. The entire teachings of these prior applications are incorporated herein by reference.

SPONSORSHIP

The present invention was supported by grant nos. R01 DK41487, R01 DK69255 and RO139255 from the National Institutes of Health, contract R43 CA 58159 from the National Cancer Institute, grant GM 35712 from the National Institute of General Medical Sciences, and by the University of Michigan Comprehensive Cancer Center grant 2P30 CA 46592 from the National Cancer Institute, U.S. Public Health Service, DHHS. Grant number for Merit Award from Veteran's Administration. The government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates generally to ceramide-like compounds and, more particularly, to ceramide-like compounds containing a tertiary amine group and their use in therapeutic methods.

BACKGROUND OF THE INVENTION

Hundreds of glycosphingolipids (GSLs) are derived from glucosylceramide (GlcCer), which is enzymatically formed from ceramide and UDP-glucose. The enzyme involved in GlcCer formation is UDP-glucose:N-acylsphingosine glucosyltransferase (GlcCer synthase). The rate of GlcCer formation under physiological conditions may depend on the tissue level of UDP-glucose, which in turn depends on the level of glucose in a particular tissue (Zador, I. Z. et al., "A Role for Glycosphingolipid Accumulation in the Renal Hypertrophy of Streptozotocin-Induced Diabetes Mellitus," *J. Clin. Invest.,* 91:797-803 (1993)). In vitro assays based on endogenous ceramide yield lower synthetic rates than mixtures containing added ceramide, suggesting that tissue levels of ceramide are also normally rate-limiting (Brenkert, A. et al., "Synthesis of Galactosyl Ceramide and Glucosyl Ceramide by Rat Brain: Assay Procedures and Changes with Age," *Brain Res.,* 36:183-193 (1972)).

It has been found that the level of GSLs controls a variety of cell functions, such as growth, differentiation, adhesion between cells or between cells and matrix proteins, binding of microorganisms and viruses to cells, and metastasis of tumor cells. In addition, the GlcCer precursor, ceramide, may cause differentiation or inhibition of cell growth (Bielawska, A. et al., "Modulation of Cell Growth and Differentiation by Ceramide," *FEBS Letters,* 307:211-214 (1992)) and be involved in the functioning of vitamin D_3, tumor necrosis factor-α, interleukins, and apoptosis (programmed cell death). The sphingols (sphingoid bases), precursors of ceramide, and products of ceramide catabolism, have also been shown to influence many cell systems, possibly by inhibiting protein kinase C (PKC).

It is likely that all the GSLs undergo catabolic hydrolysis, so any blockage in the GlcCer synthase should ultimately lead to depletion of the GSLs and profound changes in the functioning of a cell or organism. An inhibitor of GlcCer synthase, PDMP (1R-phenyl-2R-decanoylamino-3-morpholino-1-propanol), previously identified as the D-threo isomer (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.,* 28:565-571 (1987)), has been found to produce a variety of chemical and physiological changes in cells and animals (Radin, N. S. et al., "Use of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol (PDMP), an Inhibitor of Glucosylceramide Synthesis," *In NeuroProtocols, A Companion to Methods in Neurosciences*, S. K. Fisher et al., Ed., (Academic Press, San Diego) 3:145-155 (1993) and Radin, N. S. et al., "Metabolic Effects of Inhibiting Glucosylceramide Synthesis with PDMP and Other Substances," *In Advances in Lipid Research; Sphingolipids in Signaling, Part B*., R. M. Bell et al., Ed. (Academic Press, San Diego) 28:183-213 (1993)). Particularly interesting is the compound's ability to cure mice of cancer induced by Ehrlich ascites carcinoma cells (Inokuchi, J. et al., "Antitumor Activity in Mice of an Inhibitor of Glycosphingolipid Biosynthesis," *Cancer Lett.,* 38:23-30 (1987)), to produce accumulation of sphingosine and N,N-dimethylsphingosine (Felding-Habermann, B. et al., "A Ceramide Analog Inhibits T Cell Proliferative Response Through Inhibition of Glycosphingolipid Synthesis and Enhancement of N,N-Dimethylsphingosine Synthesis," *Biochemistry,* 29:6314-6322 (1990)), and to slow cell growth (Shayman, J. A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.* 266:22968-22974 (1991)). Compounds with longer chain fatty acyl groups have been found to be substantially more effective (Abe, A. et al., "Improved Inhibitors of Glucosylceramide Synthesis," *J. Biochem.,* 111:191-196 (1992)).

The importance of GSL metabolism is underscored by the seriousness of disorders resulting from defects in GSL metabolizing enzymes (which diseases may collectively be referred to as "glycosphingolipidoses"). For example, Tay-Sachs, Gaucher's, and Fabry's diseases, resulting from enzymatic defects in the GSL degradative pathway and the accumulation of GSL in the patient, all have severe clinical manifestations. Another example of the importance of GSL function is seen in a mechanism by which blood cells, whose surfaces contain selectins, can, under certain conditions, bind to GSLs in the blood vessel walls and produce acute, life-threatening inflammation (Alon, R. et al., "Glycolipid Ligands for Selectins Support Leukocyte Tethering & Rolling Under Physiologic Flow Conditions," *J. Immunol.,* 154:5356-5366 (1995)).

At present there is only one treatment available for patients with Gaucher disease, wherein the normal enzyme which has been isolated from normal human tissues or cultured cells is administered to the patient. As with any drug isolated from human material, great care is needed to prevent contamination with a virus or other dangerous substances. Treatment for an individual patient is extremely expensive, costing hundreds of thousands, or even millions of dollars, over a patient's lifetime. It would thus be desirable to provide a treatment which includes administration of a compound that is readily available and/or producible from common materials by simple reactions.

Possibly of even greater clinical relevance is the role of glucolipids in cancer. For example, it has been found that certain GSLs occur only in tumors; certain GSLs occur at abnormally high concentrations in tumors; certain GSLs,

added to tumor cells in culture media, exert marked stimulatory or inhibitory actions on tumor growth; antibodies to certain GSLs inhibit the growth of tumors; the GSLs that are shed by tumors into the surrounding extracellular fluid inhibit the body's normal immunodefense system; the composition of a tumor's GSLs changes as the tumors become increasingly malignant; and, in certain kinds of cancer, the level of a GSL circulating in the blood gives useful information regarding the patient's response to treatment. Because of the significant impact GSLs have on several biochemical processes, there remains a need for compounds having improved GlcCer synthase inhibition activity.

It would thus be desirable to provide compounds which inhibit GlcCer synthase activity, thereby lowering the level of GSLs and increasing GSL precursor levels, e.g. increasing the levels of ceramide and sphingols. It would further be desirable to provide compounds which inhibit GlcCer synthase activity and lower the level of GSLs without also increasing ceramide levels. It would also be desirable to provide compounds and therapeutic methods to treat conditions and diseases associated with altered GSL levels and/or GSL precursor levels.

SUMMARY OF THE INVENTION

Novel compounds are provided which inhibit GlcCer formation by inhibiting the enzyme GlcCer synthase, thereby lowering the level of GSLs. The compounds of the present invention have improved GlcCer synthase inhibition activity and are, therefore, highly useful in therapeutic methods for treating various conditions and diseases associated with altered GSL levels, as well as GSL precursor levels. For example, the compounds of the present invention may be useful in methods involving cancer growth and metastasis, the growth of normal tissues, the ability of pathogenic microorganisms to bind to normal cells, the binding between similar cells, the binding of toxins to human cells, and the ability of cancer cells to block the normal process of immunological cytotoxic attack.

Additional objects, advantages, and features of the present invention will become apparent from the following description and appended claims, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and subjoined claims and by referencing the following drawings in which:

FIG. **1** is a graph showing the growth and survival of 9 L gliosarcoma cells grown in medium containing different GlcCer synthase inhibitors;

FIG. **2** is a graph showing the protein content of MDCK cells cultured for 24 hr in medium containing different concentrations of the separated erythro- and threo-isomers of a preferred compound of the present invention;

FIG. **3** is a graph showing [^{3}H]thymidine incorporation into the DNA of MDCK cells treated with a preferred compound of the present invention;

FIGS. **4**A and **4**B are graphs showing the effects of P4 and p-methoxy-P4 on GlcCer synthase activity;

FIG. **5** is a graph showing the linear relationship between the inhibition of GlcCer synthase activity and electronic parameter (δ) and hydrophobic parameter (π);

FIG. **6** is a graph showing the effects of dioxy P4 derivatives on GlcCer synthase activity;

FIG. **7** is a bar graph showing the effects of D-t-3',4'-ethylenedioxy-P4 on GlcCer synthesis and cell growth;

FIG. **8** is a schematic of the synthetic pathway for 4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol;

FIG. **9** is an illustration of the structures of P4 and of phenyl-substituted P4 homologues;

FIG. **10** is an HPLC chromatogram showing the separation of the enantiomers of P4 and p-methoxy-P4 by chiral chromatography;

FIG. **11** is a graph showing the effects of D-threo-4'-hydroxy-P4 as compared to D-threo-p-methoxy-P4 on GlcCer synthase activity;

FIG. **12** is a graph showing the effects of D-threo enantiomers of P4,4'-hydroxy-P4 and 3',4'-ethylenedioxy-P4 on 1-O-acyceramide synthase activity;

FIG. **13** is a graph showing the effect of D-threo-P4 on GlcCer synthesis and cell growth;

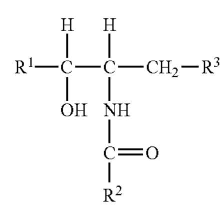
FIG. **14** is a graph showing the effect of D-threo-4'-hydroxy-P4 on GlcCer synthesis and cell growth; and

FIG. **15** is a graph showing the effect of D-threo-3',4'-ethylenedioxy-P4 on GlcCer synthesis and cell growth.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Novel compounds are provided which inhibit GlcCer formation by inhibiting the enzyme GlcCer synthase, thereby lowering the level of GSLs. The compounds of the present invention have improved GlcCer synthase inhibitory activity and are, therefore, highly useful in therapeutic methods for treating various conditions and diseases associated with altered GSL levels.

The compounds of the present invention generally have the following formula:



wherein

R^1 is a phenyl group, preferably a substituted phenyl group such as p-methoxy, hydroxy, dioxane substitutions such as methylenedioxy, ethylenedioxy, and trimethylenedioxy, cyclohexyl or other acyclic group, t-butyl or other branched aliphatic group, or a long alkyl or alkenyl chain, preferably 7 to 15 carbons long with a double bond next to the kernel of the structure. The aliphatic chain can have a hydroxyl group near the two asymmetric centers, corresponding to phytosphingosine.

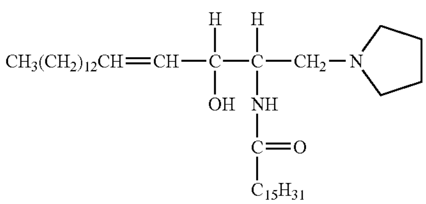
R^2 is an alkyl residue of a fatty acid, 2 to 18 carbons long. The fatty acid can be saturated or unsaturated, or possess a small substitution at the C-2 position (e.g., a hydroxyl group). It is contemplated that the R^2 group fatty acid may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 carbons long. Longer fatty acids also may be useful. Preferably R^2 in the above structure is either 5 carbons or 7 carbons in length.

R^3 is a tertiary amine, preferably a cyclic amine such as pyrrolidine, azetidine, morpholine or piperidine, in which the nitrogen atom is attached to the kernel (i.e., a tertiary amine).

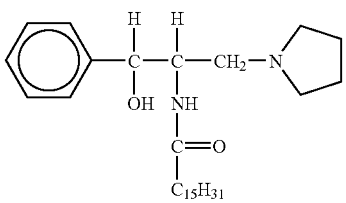
All four structural isomers of the compounds are contemplated within the present invention and may be used either singly or in combination (i.e., DL-threo or DL-erythro).

The preferred aliphatic compound of the present invention is D-threo-1-pyrrolidino-1-deoxyceramide, identified as IV-231B herein and also referred to as PD. The preferred aromatic compound of the present invention is 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, identified as BML-119 herein and also referred to as P4. The structures of the preferred compounds are as follows:

PD



P4



Additional preferred compounds of the present invention are D-t-3',4'-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, also referred to herein as D-t-3',4'-ethylenedioxy-P4, and D-t-4'-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol, also referred to herein as D-t-4'-hydroxy-P4.

By increasing the acyl chain length of PDMP from 10 to 16 carbon atoms, the efficacy of the compounds of the present invention as GlcCer synthase inhibitors is greatly enhanced. The use of a less polar cyclic amine, especially a pyrrolidine instead of a morpholine ring, also increases the efficacy of the compounds. In addition, replacement of the phenyl ring by a chain corresponding to sphingosine yields a strongly inhibitory material. By using a chiral synthetic route, it was discovered that the isomers active against GlcCer synthase had the R,R-(D-threo)-configuration. However, strong inhibition of the growth of human cancer cells in plastico was produced by both the threo and erythro racemic compounds, showing involvement of an additional factor beyond simple depletion of cell glycosphingolipids by blockage of GlcCer synthesis. The growth arresting effects could be correlated with increases in cellular ceramide and diglyceride levels.

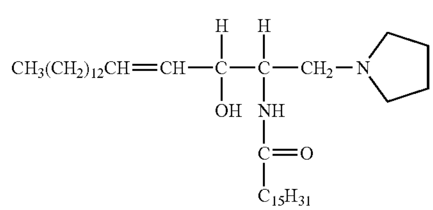
Surprisingly, the aliphatic pyrrolidino compound of the present invention (identified as IV-231B), was strongly inhibitory toward the GlcCer synthase and produced almost complete depletion of glycolipids, but did not inhibit growth or cause an accumulation of ceramide. Attempts were made to determine if the differences in growth effects could be attributed to the influence of the inhibitors on related enzymes (ceramide and sphingomyelin synthase and ceramidase and sphingomyelinase). While some stimulation or inhibition of enzyme activity was noted, particularly at high inhibitor concentrations (50 μM), these findings did not explain the differing effects of the different inhibitors.

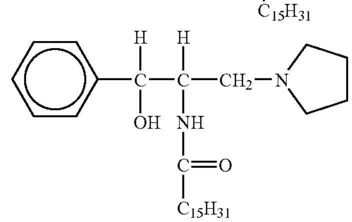
By slowing the synthesis of GlcCer, the compounds of the present invention lower the levels of all the GlcCer-derived GSLs due to the GSL hydrolases which normally destroy them. While the body will continue to make the more complex GSLs from available GlcCer, the rate of synthesis will slow down as the level of GlcCer diminishes. The rate of lowering depends on the normal rate of destruction of each GSL. These rates, however, are relatively rapid in animals and cultured cells.

At higher dosages, many of the compounds of the present invention produce an elevation in the level of ceramide. Presumably this occurs because cells continue to make ceramide despite their inability to utilize it for GlcCer synthesis. Ceramide is also normally converted to sphingomyelin, but this process does not seem to be able to handle the excess ceramide. It has been unexpectedly found, however, that an additional process is also involved, since even those isomers that are inert against GlcCer synthase also produce an elevation in ceramide levels. Moreover, the blockage of GlcCer synthase can occur at low inhibitor dosages, yet ceramide accumulation is not produced. The preferred aliphatic compound of the present invention, D-threo-1-pyrrolidino-1-deoxyceramide (PD), does not produce ceramide accumulation at all, despite almost complete blockage of GlcCer synthesis.

This distinction between the aromatic and the aliphatic compounds of the present invention is important because ceramide has recently been proposed to cause cell death (apoptosis) by some still unknown mechanism. At lower dose levels, the aromatic compounds of the present invention cause GSL disappearance with only small accumulation of ceramide and inhibition of cell growth. Higher dosages cause much more ceramide deposition and very slow cell growth or cell death.

In certain embodiments, the inventors found that compounds containing a 16 carbon fatty acyl group is an extremely efficient and potent GlcCer synthase inhibitor. However, the longer the acyl chain of the PDMP-based compounds, the more lipophilic the agent. The inventors found that the C16 fatty acyl PDMP derivatives had a long retention time within the body. In some instances, it may be desirable to produce compounds having a C6 or C8 fatty acyl chain (i.e., R^2 in the above structures is a C5 or C7 fatty acyl chain backbone). Specifically contemplated by the present invention are compounds of the following formulas:





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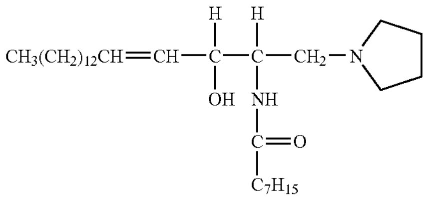
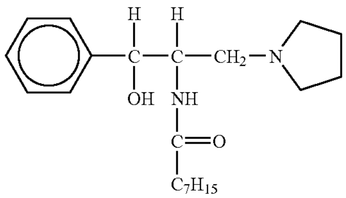




In one embodiment of the present invention, methods of treating patients suffering from inborn genetic errors in the metabolism of GlcCer and its normal anabolic products (lactosylceramide and the more complex GSLs) are provided. The presently known disorders in this category include Gaucher, Fabry, Tay-Sachs, Sandhoff, and GM1 gangliosidosis. The genetic errors lie in the patient's inability to synthesize a hydrolytic enzyme having normal efficiency. Their inefficient hydrolase allows the GSL to gradually accumulate to a toxic degree, debilitating or killing the victim. The compounds of the present invention slow the formation of GSLs, thus allowing the defective hydrolase to gradually "catch up" and restore the concentrations of GSLs to their normal levels and thus the compounds may be administered to treat such patients.

With respect to Gaucher disease, it has been calculated that much of the patient's accumulated GlcCer in liver and spleen arises from the blood cells, which are ultimately destroyed in these organs after they have reached the end of their life span. The actual fraction, lipid derived from blood cells versus lipid formed in the liver and spleen cells, is actually quite uncertain, but the external source must be important. Therefore, it is necessary for the compounds of the present invention to deplete the blood cells as they are formed or (in the case of white blood cells) while they still circulate in the blood. Judging from toxicity tests, the white cells continue to function adequately despite their loss of GSLs. Although the toxicity studies were not of a long enough duration to produce many new red cells with low GSL content, it is possible that circulating red cells also undergo turnover (continual loss plus replacement) of GSLs.

In an alternative embodiment of the present invention, for the treatment of disorders involving cell growth and division, high dosages of the compounds of the present invention are administered but only for a relatively short time. These disorders include cancer, collagen vascular diseases, atherosclerosis, and the renal hypertrophy of diabetic patients. Accumulation or changes in the cellular levels of GSLs have been implicated in these disorders and blocking GSL biosynthesis would allow the normal restorative mechanisms of the body to resolve the imbalance.

With atherosclerosis, it has been shown that arterial epithelial cells grow faster in the presence of a GlcCer product (lactosylceramide). Oxidized serum lipoprotein, a material that normally circulates in the blood, stimulates the formation of plaques and lactosylceramide in the inner lining of blood vessels. Treatment with the compounds of the present invention would inhibit this mitogenic effect.

In an additional embodiment of the present invention, patients suffering from infections may be treated with the compounds of the present invention. Many types of pathogenic bacteria have to bind to specific GSLs before they can induce their toxic effects. As shown in Svensson, M. et al., "Epithelial Glucosphingolipid Expression as a Determinant of Bacterial Adherence and Cytokine Production," *Infect. and Immun.,* 62:4404-4410 (1994), expressly incorporated by reference, PDMP treatment reduces the adherence of *E. coli* to mammalian cells. Several viruses, such as influenza type A, also must bind to a GSL. Several bacterial toxins, such as the verotoxins, cannot themselves act without first binding to a GSL. Thus, by lowering the level of GSLs, the degree of infection may be ameliorated. In addition, when a patient is already infected to a recognizable, diagnosable degree, the compounds of the present invention may slow the further development of the infection by eliminating the binding sites that remain free.

It has been shown that tumors produce substances, namely gangliosides, a family of GSLs, that prevent the host i.e., patient, from generating antibodies against the tumor. By blocking the tumor's ability to secrete these substances, antibodies against the tumor can be produced. Thus, by administering the GlcCer synthase inhibitors of the present invention to the patient, the tumors will become depleted of their GSLs and the body's normal immunological defenses will come into action and destroy the tumor. This technique was described in Inokuchi, J. et al., "Antitumor Activity in Mice of an Inhibitor of Glycosphingolipid Biosynthesis," *Cancer Lett.,* 38:23-30 (1987), expressly incorporated by reference. The compounds of the present invention and in particular the aliphatic compounds require much lower doses than those previously described. This is particularly important because the lower dose may reduce certain side effects. Moreover, because the aliphatic compounds of the present invention do not produce ceramide accumulation, they are less toxic. In addition, 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4), may act via two pathways, GSL depletion and ceramide accumulation.

In an alternative embodiment, a vaccine-like preparation is provided. Here, cancer cells are removed from the patient (preferably as completely as possible), and the cells are grown in culture in order to obtain a large number of the cancer cells. The cells are then exposed to the inhibitor for a time sufficient to deplete the cells of their GSLs (generally 1 to 5 days) and are reinjected into the patient. These reinjected cells act like antigens and are destroyed by the patient's immunodefense system. The remaining cancer cells (which could not be physically removed) will also be attacked by the patient's antibodies. In a preferred embodiment, the patient's circulating gangliosides in the plasma are removed by plasmapheresis, since the circulating gangliosides would tend to block the immunodefense system.

It is believed that tumors are particularly dependent on GSL synthesis for maintenance of their growth (Hakomori, S. "New Directions in Cancer Therapy Based on Aberrant Expression of Glycosphingolipids: Anti-adhesion and Ortho-Signaling Therapy," Cancer Cells 3:461-470 (1991)). Accumulation of ceramide in treated tumors also slows their growth or kills them. Tumors also generate large amounts of GSLs and secrete them into the patient's body, thereby preventing the host's normal response by immunoprotective cells, which should generate antibodies against or otherwise destroy tumor cells (e.g., tumors are weakly antigenic). It has also been shown that GSL depletion blocks the metastasis of tumor cells (Inokuchi, J. et al., "Inhibition of Experimental Metastasis of Murine Lewis Long Carcinoma by an

Inhibitor of Glucosylceramide Synthase and its Possible Mechanism of Action," *Cancer Res.,* 50:6731-6737 (1990). Tumor angiogenesis (e.g., the production of blood capillaries) is strongly influenced by GSLs (Ziche, M. et al., "Angiogenesis Can Be Stimulated or Repressed in In Vivo by a Change in GM3:GD3 Ganglioside Ratio," *Lab. Invest.,* 67:711-715 (1992)). Depleting the tumor of its GSLs should block the tumors from generating the new blood vessels they need for growth.

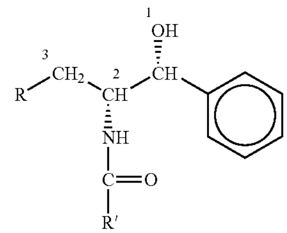
A further important characteristic of the compounds of the present invention is their unique ability to block the growth of multidrug resistant ("MDR") tumor cells even at much lower dosages. This was demonstrated with PDMP by Rosenwald, A. G. et al., "Effects of the Glycosphingolipid Synthesis Inhibitor, PDMP, on Lysosomes in Cultured Cells," *J. Lipid Res.,* 35:1232 (1994), expressly incorporated by reference. Tumor cells that survive an initial series of therapeutic treatments often reappear some years later with new properties—they are now resistant to a second treatment schedule, even with different drugs. This change has been attributed to the appearance in the tumor of large amounts of a specific MDR protein (P-glycoprotein). It has been suggested that protein kinase C (PKC) may be involved in the action or formation of P-glycoprotein (Blobe, G. C. et al., "Regulation of PKC and Its Role in Cancer Biology," *Cancer Metastasis Rev.,* 13:411-431 (1994)). However, decreases in PKC have other important effects, particularly slowing of growth. It is known that PDMP does lower the cellular content of PKC (Shayman, J. A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.,* 266:22968-22974 (1991)) but it is not clear why it so effectively blocks growth of MDR cells (Rosenwald, A. G. et al., "Effects of the Glycosphingolipid Synthesis Inhibitor, PDMP, On Lysosomes in Cultured Cells," *J. Lipid Res.,* 35:1232 (1994)). A recent report showed that several lipoidal amines that block MDR action also lower the level of the enzyme acid sphingomyelinase (Jaffrezou, J. et al., "Inhibition of Lysosomal Acid Sphingomyelinase by Agents which Reverse Multidrug Resistance," *Biochim. Biophys. Acta,* 1266:1-8 (1995)). One of these agents was also found to increase the cellular content of sphingosine 5-fold, an effect seen with PDMP as well. One agent, chlorpromazine, behaves like the compounds of the present invention, in its ability to lower tissue levels of GlcCer (Hospattankar, A. V. et al., "Changes in Liver Lipids After Administration of 2-Decanoylamino-3-Morpholinopropiophenone and Chlorpromazine," *Lipids,* 17:538-543 (1982)).

It will be appreciated by those skilled in the art that the compounds of the present invention can be employed in a wide variety of pharmaceutical forms; the compound can be employed neat or admixed with a pharmaceutically acceptable carrier or other excipients or additives. Generally speaking, the compound will be administered orally or intravenously. It will be appreciated that therapeutically acceptable salts of the compounds of the present invention may also be employed. The selection of dosage, rate/frequency and means of administration is well within the skill of the artisan and may be left to the judgment of the treating physician or attending veterinarian. The method of the present invention may be employed alone or in conjunction with other therapeutic regimens. It will also be appreciated that the compounds of the present invention are also useful as a research tool e.g. to further investigate GSL metabolism.

The following Specific Example further describes the compounds and methods of the present invention.

SPECIFIC EXAMPLE 1

The following formulas set forth preferred aromatic and aliphatic compounds:

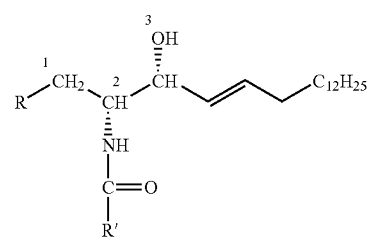


identified as (1R,2R)-1-phenyl-2-acylamino-3-cyclic amino-1-propanol, and referred to herein as the "aromatic inhibitors," wherein

The phenyl group can be a substituted phenyl group (such as p-methoxyphenyl).

R' is an alkyl residue of a fatty acid, 2 to 18 carbons long. The fatty acid can be saturated or unsaturated, or possess a small substitution at the C-2 position (e.g., a hydroxyl group). It is contemplated that the R' group fatty acid may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 carbons long. Longer fatty acids also may be useful. Preferrably R' in the above structure is either 5 carbons or 7 carbons in length.

R is morpholino, pyrrolidino, piperidino, azetidino (trimethyleneimino), N-methylethanolamino, diethylamino or N-phenylpiperazino. A small substituent, such as a hydroxyl group, is preferably included on the cyclic amine moiety.



identified as (2R,3R)-2-palmitoyl-sphingosyl amine or 1-cyclic amino-1-deoxyceramide or 1-cyclic amino-2-hexadecanoylamino-3-hydroxy-octadec-4,5-ene, and referred to herein as the "aliphatic inhibitors," wherein

R' is an alkyl residue of a fatty acid, 2 to 18 carbons long. The fatty acid can be saturated or unsaturated, or possess a small substitution at the C-2 position (e.g., a hydroxyl group). It is contemplated that the R' group fatty acid may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 carbons long. Longer fatty acids also may be useful. Preferrably R' in the above structure is either 5 carbons or 7 carbons in length.

R is morpholino, pyrrolidino, piperidino, azetidino (trimethyleneimino), N-methylethanolamino, diethylamino or N-phenylpiperazino. A small substituent, such as a hydroxyl group, is preferably included on the cyclic amine moiety.

The long alkyl chain shown in Formula II can be 8 to 18 carbon atoms long, with or without a double bond near the asymmetric carbon atom (carbon 3). Hydroxyl groups can, with advantage, be substituted along the aliphatic chain,

particularly on carbon 4 (as in the naturally occurring sphingol, phytosphingosine). The long chain can also be replaced by other aliphatic groups, such at t-butyl or cyclopentyl.

The aromatic inhibitors (see Formula I and Table 1) were synthesized by the Mannich reaction from 2-N-acylaminoacetophenone, paraformaldehyde, and a secondary amine as previously described (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.,* 28:565-571 (1987) and Vunnam, R. R. et al., "Analogs of Ceramide that Inhibit Glucocerebroside Synthetase in Mouse Brain," *Chem. Phys. Lipids,* 26:265-278 (1980)). For those syntheses in which phenyl-substituted starting materials were used, the methyl group in the acetophenone structure was brominated and converted to the primary amine. Bromination of p-methoxyacetophenone was performed in methanol. The acetophenones and amines were from Aldrich Chemical Co., St. Louis, Mo. Miscellaneous reagents were from Sigma Chemical Co. and the sphingolipids used as substrates or standards were prepared by methods known in the art. The reactions produce a mixture of four isomers, due to the presence of two asymmetric centers.

The aliphatic inhibitors (See Formula II and Table 2) were synthesized from the corresponding 3-t-butyldimethylsilyl-protected sphingols, prepared by enantioselective aldol condensation (Evans, D. A. et al., "Stereoselective Aldol Condensations Via Boron Enolates," *J. Am. Chem. Soc.,* 103: 3099-3111 (1981) and Abdel-Magid, A. et al., "Metal-Assisted Aldol Condensation of Chiral A-Halogenated Imide Enolates: A Stereocontrolled Chiral Epoxide Synthesis," *J. Am. Chem. Soc.,* 108:4595-4602 (1986)) using a modification of the procedure of Nicolaou et al. (Nicolaou, K. C. et al., "A Practical and Enantioselective Synthesis of Glycosphingolipids and Related Compounds. Total Synthesis of Globotriaosylceramide (Gb_3)," *J. Am. Chem. Soc.,* 110:7910-7912 (1988)). Each protected sphingol was first converted to the corresponding primary triflate ester, then reacted with a cyclic amine. Subsequent N-acylation and desilylation led to the final products in good overall yield (Carson, K. G. et al., "Studies on Morpholinosphingolipids: Potent Inhibitors of Glucosylceramide Synthase," *Tetrahedron Lett.,* 35:2659-2662 (1994)). The compounds can be called 1-morpholino-(or pyrrolidino)-1-deoxyceramides.

Labeled ceramide, decanoyl sphingosine, was prepared by reaction of the acid chloride and sphingosine (Kopaczyk, K. C. et al., "In Vivo Conversions of Cerebroside and Ceramide in Rat Brain," *J. Lipid Res.,* 6:140-145 (1965)) and NBD-SM (12-[N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]-sphingosylphosphorylcholine) was from Molecular Probes, Inc., Eugene, Oreg.

Methods

TLC of the amines was carried out with HPTLC plates (E. Merck silica gel 60) and C-M-HOAc 90:10:10 (solvent A) or 85:15:10 (solvent B) or C-M-conc. ammonium hydroxide 30:10:1 (solvent C). The bands were stained with iodine or with Coomassie Brilliant Blue R-250 (Nakamura, K. et al., "Coomassie Brilliant Blue Staining of Lipids on Thin-Layer Plates," *Anal. Biochem.,* 142:406-41 (1984)) and, in the latter case, quantified with a Bio-Rad Model 620 videodensitometer operated with reflected white light. The faster band of each PDMP analog, previously identified as the erythro form, corresponds to the 1S,2R and 1R,2S stereoisomers, and the slower band, previously identified as the threo form, corresponds to the 1R,2R and 1S,2S stereoisomers.

TLC of the cell lipids was run with C-M-W 24:7:1 (solvent D) or 60:35:8 (solvent E).

Growth of cell lines. Comparisons of different inhibitors with regard to suppression of human cancer cell growth were made by the University of Michigan Cancer Center in vitro Drug Evaluation Core Laboratory. MCF-7 breast carcinoma cells, HT-29 colon adenocarcinoma cells, H-460 lung large cell carcinoma cells, and 9 L brain gliosarcoma cells were grown in RPMI 1640 medium with 5% fetal bovine serum, 2 mM glutamine, 50 units/ml of penicillin, 50 mg/ml of streptomycin, and 0.1 mg/ml of neomycin. UMSCC-10A head and neck squamous carcinoma cells were grown in minimal essential medium with Earle salts and the same supplements. Medium components were from Sigma Chemical Co. Cells were plated in 96-well microtiter plates (1000 cells/well for H-460 and 9 L cells, and 2000 cells/well for the other lines), and the test compounds were added 1 day later. The stock inhibitor solutions, 2 mM in 2 mM BSA, were diluted with different amounts of additional 2 mM BSA, then each solution was diluted 500-fold with growth medium to obtain the final concentrations indicated in the Figures and Tables.

Five days after plating the H-460 and 9 L cells, or 6 days for the other lines, cell growth was evaluated by staining the adhering cells with sulforhodamine B and measuring the absorbance at 520 nm (Skehan, P. et al., "New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening," *J. Natl. Cancer Inst.,* 82:1107-1112 (1990)). The absorbance of the treated cultures is reported as percent of that of control cultures, to provide an estimate of the fraction of the cells that survived, or of inhibition of growth rate.

For the experiments with labeled thymidine, each 8.5 cm dish contained 500,000 Madin-Darby canine kidney (MDCK) cells in 8 ml of Dulbecco modified essential supplemented medium. The cells were incubated at 37° C. in 5% CO_2 for 24 h, then incubated another 24 h with medium containing the inhibitor-BSA complex. The control cells were also incubated in the presence of BSA. The cells were washed with phosphate/saline and trichloroacetic acid, then scraped off the dishes, dissolved in alkali, and analyzed for protein and DNA incorporated tritium. [Methyl-^{3}H]thymidine (10 μCi) was added 4 h prior to harvesting.

Assay of sphingolipid enzymes. The inhibitors were evaluated for their effectiveness against the GlcCer synthase of MDCK cell homogenates by incubation in a thermostatted ultrasonic bath (Radin N. S. et al., "Ultrasonic Baths as Substitutes for Shaking Incubator Baths," *Enzyme,* 45:67-70 (1991)) with octanoyl sphingosine and uridinediphospho [^{3}H]glucose (Shukla, G. S. et al., "Glucosylceramide Synthase of Mouse Kidney: Further Characterization and Improved Assay Method," *Arch. Biochem. Biophys.,* 283: 372-378 (1990)). The lipoidal substrate (85 μg) was added in liposomes made from 0.57 mg dioleoylphosphatidylcholine and 0.1 mg of Na sulfatide. Confluent cells were washed, then homogenized with a micro-tip sonicator at 0° C. for 3×30 sec; ~0.2 mg of protein was used in each assay tube. In the case of the aromatic inhibitors, the test compound was simply evaporated to dryness from solution in the incubation tube. This method of adding the inhibitor was found to give the same results as addition as a part of the substrate liposomes. The aliphatic inhibitors, which appeared to be less soluble in water, were added as part of the substrate liposomes.

Acid and neutral ceramidases were assayed under conditions like those above, but the medium contained 110 μM

[1-^{14}C]decanoyl sphingosine (10^5 cpm) in 340 μM dioleoylphosphatidylcholine liposomes and 0.34 mg of MDCK cellular protein homogenate. The acid enzyme was incubated in 32.5 mM citrate-Na^+ (pH 4.5) and the neutral enzyme buffer was 40 mM Tris-Cl^- (pH 7.1 at 37° C.). After 60 min in the ultrasonic bath, 3 ml of C-M 2:1, carrier decanoic acid, and 0.6 ml of 0.9% saline were added and the lipids in the lower layer were separated by TLC with C-HOAc 9:1. The liberated decanoic acid was scraped off the glass plate and counted.

Ceramide synthase was assayed with 1 μM [3-^{3}H]sphingosine (70,000 cpm, repurified by column chromatography), 0.2 mM stearoyl-CoA, 0.5 mM dithiothreitol, and ~300 μg of MDCK homogenate protein in 25 mM phosphate-K^+ buffer, pH 7.4, in a total volume of 0.2 ml. The incubation (for 30 min) and TLC were carried out as above and the ceramide band was counted.

Sphingomyelin synthase was evaluated with 44 μM [^{14}C] decanoyl sphingosine (10^5 cpm) dispersed with 136 μM dioleoyllecithin as in the ceramide synthase assay, and 5 mM EDTA and 50 mM Hepes-Na^+ pH 7.5, in a total volume of 0.5 ml. MDCK homogenate was centrifuged at 600×g briefly, then at 100,000×g for 1 h, and the pellet was suspended in water and sonicated with a dipping probe. A portion of this suspension containing 300 μg of protein was used. Incubation was at 37° C. for 30 min, after which the lipids were treated as above, using C-M-W 60:35:8 for the isolation of the labeled decanoyl SM.

Acid and neutral SMase assays were based on the procedures of Gatt et al. (Gatt, S. et al., "Assay of Enzymes of Lipid Metabolism With Colored and Fluorescent Derivatives of Natural Lipids," *Meth. Enzymol.*, 72:351-375 (1981)), using liposomes containing NBD-SM dispersed like the labeled ceramide (10 μM substrate and 30 μM lecithin). The assay medium for the neutral enzyme also contained 50 mM Tris-Cl^- (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$ and 0.29 mg of MDCK cell protein in a total volume of 0.25 ml. Incubation was at 37° C. for 30 min in the ultrasonic bath, then the fluorescent product, NBD-ceramide, was isolated by partitioning the assay mixture with 0.45 ml 2-propanol, 1.5 ml heptane, and 0.2 ml water. After centrifugation, a trace of contaminating NBD-SM was removed from 0.9 ml of the upper layer by washing with 0.35 ml water. The upper layer was analyzed with a fluorometer (460 nm excitation, 515 nm emission).

Acid SMase was assayed with the same liposomes in 0.2 ml of assay mixture containing 125 mM NaOAc (pH 5.0) and 61 μg of cell protein, with 60 min of incubation at 37° C. The resultant ceramide was determined as above.

Results

Table 1 lists the aromatic compounds (see Formula I) synthesized and their migration rates on silica gel TLC plates. Separation of the threo- and erythro-steroisomers by TLC was generally very good, except for BML-120, -121, and -122 in the acidic solvent. In the basic solvent BML-119 and BML-122 yielded poorly resolved double bands. BML-112 was unexpectedly fast-running, especially when compared with BML-120; both are presumably dihydrochlorides.

TABLE 1

STRUCTURES OF THE AROMATIC INHIBITORS

BML Number or Name	R Group	Phenyl Substituent	TLC hR_1 Value[a]
PDMP[b]	morpholino		34(47)
PPMP	morpholino		(53)
112	N-phenylpiperazino		56
113	morpholino	p-fluoro	25
114	diethylamino		25
115	piperidino (pentamethyleneimino)		29
116	hexamethyleneimino		34
117[b]	morpholino	p-fluoro	41
118	piperidino	p-fluoro	26
119	pyrrolidino (tetramethyleneimino)		20–70(44)
120	1-methylpiperazino		7–62
121	3-dimethylaminopiperidino		1–30
122	N-methylethanolamino		6–71
123	azetidino (trimethyleneimino)		12
124	amino		15
125	morpholino	p-methoxy	37
126	pyrrolidino	p-methoxy	(50)

[a]Only the relative R_f value of the faster-moving band is shown. The first value was obtained with solvent A, the second with solvent C, and the numbers in parentheses, with solvent B. In the case of BML-117, −125, and −126, a 20-cm high TLC plate was used to improve the separation.
[b]The fatty acid chain suggested by the R' group is decanoyl, not palmitoyl.

Table 2 describes four aliphatic inhibitors (see Formula II), which can be considered to be ceramide analogs in which the C-1 hydroxyl group is replaced by a cyclic amine. It should be noted that the carbon frameworks of compounds in Tables 1 and 2 are numbered differently (see Formulas I and II), thus affecting comparisons of stereochemical configurations. The threo- and erythro-isomers separated very poorly on TLC plates. Like the aromatic inhibitors, however, the morpholine compounds ran faster than the pyrrolidine compounds. The latter are presumably more strongly adsorbed by the silica gel because they are more basic.

TABLE 2

CHARACTERIZATION OF THE SPHINGOSYL INHIBITORS

Number	R Group	Sphingol Structure	TLC hR_f Value[a]
IV-181A	morpholino	2R, 3S	43
IV-206A	morpholino	2R, 3R	40
IV-230A	pyrrolidino	2R, 3S	31
IV-231B	pyrrolidino	2R, 3R	31

[a]TLC solvent: C-M-HOAc 90:5:10. Similar but faster migrations were obtained with solvent A.

Structure-activity correlations. The results of testing the compounds in an assay system for GlcCer synthase are listed in Table 3. Each inhibition determination (±SD) shown in Table 3 was carried out in triplicate. Some of the inhibitors were tested as mixtures of DL-erythro- and DL-threo-isomers (see column 4). Only the D-threo enantiomer in each mixture was predicted to be the actual enzyme inhibitor (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.*, 28:565-571 (1987)); the content of this isomer was calculated by measuring the proportions of the threo- and erythro-racemic mixtures by quantitative TLC. The DL-threo contents were found to be in the range of 40 to 72%. The

comparisons, in the case of the mixtures, are therefore approximate (most of the samples were not purified to remove the three less-active isomers and the observed data were not corrected for the level of the primary enantiomers). The separation of the threo- and erythro-forms is most conveniently accomplished by crystallization, but the specific conditions vary for each substance; thus only BML-119, a strong inhibitor, was separated into its threo- and erythro-forms. BML-112 is not included in Table 3 because it had no inhibitory activity against GlcCer synthase of rabbit liver microsomes.

TABLE 3

Inhibition of Ceramide Glucosyltransferase of MDCK cell Homogenates by Different Compounds

Inhibitor Number	% Inhibition at 80 μM	Inhibition at 5 μM	Active Isomer[h]
BML-113	60 ± 4.7[a]		29
BML-114	31 ± 2.9[a]		20
BML-115	84 ± 0.8[a]	12.4 ± 0.7[f]	27
	82 ± 0.3[b]		
BML-116	28 ± 3.2[a]		27
BML-117	35 ± 0.6[b]		36
BML-118	62 ± 0.4[b]	8.3 ± 1.4[f]	32
BML-119	94 ± 1.4[b]	51 ± 2.3[e]	29
	97 ± 0.1[c]	49 ± 0.8[f]	
	96 ± 0.1[d]		
BML-120	11 ± 3.0[c]		26
BML-121	11 ± 0.4[c]		28
BML-122	58 ± 1.6[d]		26
BML-123	86 ± 0.1[d]	15 ± 0.8[f]	33
BML-124	−2 ± 1.6[d]		15
BML-125		9 ± 3.0[e]	26
BML-126	60 ± 1.8[e]	54 ± 0.3[f]	34
PDMP	90 ± 0.8[a]	16 ± 1.8[f]	100
PPMP		32 ± 1.8[e]	100
		32 ± 0.7[f]	
IV-181A		12 ± 0.2[g]	100
IV-206A		73 ± 1.5[g]	100
IV-230A		19 ± 2.1[g]	100
IV-231B		87 ± 0.4[g]	100

[a-g]Different samples were assayed as parts of different experiments.
[h]Percent of the active D-stereoisomer in the synthesized sample, estimated by scanning the two stained bands, assuming the slower one was the (racemic) active form.

Comparison of PDMP (1R,2R-decanoate) and PPMP (1R, 2R-palmitate), when evaluated at the same time in Expt. f, shows that an increase in the chain length of the N-acyl group from 10 to 16 carbon atoms distinctly improved the inhibitory activity against GlcCer synthase, as noted before (Abe, A. et al., "Improved Inhibitors of Glucosylceramide Synthesis," *J. Biochem.,* 111:191-196 (1992)). Accordingly, most of the other compounds were synthesized with the palmitoyl group for comparison with PPMP. The comparisons between the best inhibitors are clearer at the 5 μM level.

Replacing the oxygen in the morpholine ring of PPMP with a methylene group (BML-115) improved activity ~1.4-fold (calculated from the inhibitions at 5 μM in Expt. f and relative purities, and assuming that the percent inhibition is proportional to concentration in this region: $12.4/27\times100/32=1.4$). Previous comparison with mouse brain, human placenta, and human Gaucher spleen glucosyltransferase also showed that replacing the morpholino ring with the piperidino ring in a ketone analog of PDMP (1-phenyl-2-decanoylamino-3-piperidino-1-propanone) produced a much more active inhibitor (Vunnam, R. R. et al., "Analogs of Ceramide that Inhibit Glucocerebroside Synthetase in Mouse Brain," *Chem. Phys. Lipids,* 26:265-278 (1980)).

Replacing the piperidine group with a 7-membered ring (BML-116) greatly decreased the activity, while use of a 5-membered ring (BML-119) quadrupled the effectiveness (50 vs 12.4% inhibition). A 4-membered ring (BML-123) yielded a compound about as effective as the piperidino compound. The parent amine (BML-124), its N,N-diethyl analog (BML-114), and the sterically bulky N-phenylpiperazine analog (BML-112) displayed little or no activity.

Replacing a hydrogen atom with a fluorine atom in the p-position of the phenyl ring decreased the inhibitory power (BML-117 vs PDMP and BML-118 vs BML-115). Substitution of the p-position with an electron-donating moiety, the methoxy group, had a similar weakening effect in the case of the morpholino compound (BML-125 vs PPMP). Comparison of the pyrrolidino compounds, which are more basic than the morpholino compounds, showed that the methoxy group enhanced the inhibitory power (BML-126 vs BML-119).

Preparations of BML-119 were separated into threo and erythro racemic mixtures by HPLC on a Waters Microbondapak C_{18} column, using M-W-conc. NH_4OH 90:10:0.2 as the elution solvent. The material eluting earlier (but migrating more slowly on a TLC plate) was called BML-130; the later eluting material (faster by TLC) was called BML-129. Assay of GlcCer synthase with each preparation at 5 μM showed 15% inhibition by BML-129 and 79% inhibition by BML-130. TLC analysis of the two preparations revealed incomplete separation, which could explain the minor inhibition by BML-129. When the two stereoisomers were separated by preparative TLC, the difference in effectiveness was found to be somewhat higher, evidently due to the better separation by this method. Thus, the slower-migrating stereoisomer accounted for all or nearly all of the inhibitory activity, as noted with PDMP (Inokuchi, J. et al., "Preparation of the Active Isomer of 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol, Inhibitor of Glucocerebroside Synthetase," *J. Lipid Res.,* 28:565-571 (1987)).

Comparison of the two pairs of aliphatic inhibitors (bottom of Table 3) showed that the 2R,3R (D-threo) form is the primary inhibitor of glucosyltransferase. This finding is in agreement with previous identification of the active PDMP isomer as being the D-threo enantiomer. However, unlike the aromatic analog, BML-129 (2R,3S/2S,3R), there was a relatively small but significant activity in the case of the (erythro) 2R,3S stereoisomer. The erythro form of PDMP was found to inhibit cell proliferation of rabbit skin fibroblasts almost as well as R,R/S,S-PDMP but it did not act on the GSLs (Uemura, K. et al., "Effect of an Inhibitor of Glucosylceramide Synthesis on Cultured Rabbit Skin Fibroblasts," *J. Biochem.*, (Tokyo) 108:525-530 (1990)). As noted with the aromatic analogs, the pyrrolidine ring was more effective than the morpholine ring (Table 3).

Comparison of the aliphatic and corresponding aromatic inhibitors can be made in the case of the optically active morpholine compounds PPMP and IV-206A, both of which have the R,R structure and the same fatty acid. Here it appears that the aliphatic compound is more effective (Table 3). However, in a second comparison, at lower concentrations with the inhibitors incorporated into the substrate liposomes, the degree of inhibition was 77±0.9% with 3 μM IV-231B and 89±0.6% with 6 μM DL-threo BML-119.

Evaluations of cultured cell growth. Exposure of five different cancer cell lines to inhibitors at different concentrations for 4 or 5 days showed that the six BML compounds most active against GlcCer synthase were very effective growth inhibitors (Table 4). The IC_{50} values (rounded off to one digit in the table) ranged from 0.7 to 2.6 μM.

TABLE 4

Inhibition of Tumor Cell Growth In Vitro by Various Inhibitors

CELL TYPE	BML-115	BML-118	BML-119	BML-123	BML-126	BML-129	BML-130
MCF-7	2	2	2	2	1	3	2
H-460	2	2	1	1	1	2	3
HT-29	2		1	2	1	2	2
9L	2	2	1	2	2	2	2
UMSC C-10A	1		1	1	1	2	2

FIG. **1** shows growth and survival of 9 L gliosarcoma cells grown in medium containing different GlcCer synthase inhibitors, as described above. The BML compounds were used as synthesized (mixtures of DL-threo and -erythro stereoisomers) while the PDMP and PPMP were optically resolved R,R isomers. The concentrations shown are for the mixed racemic stereoisomers, since later work (Table 4) showed that both forms were very similar in effectiveness. FIG. **1** illustrates the relatively weak effectiveness of R,R-PPMP and even weaker effectiveness of R,R-PDMP. The three new compounds, however, are much better inhibitors of GlcCer synthase and growth. These differences in growth inhibitory power correlate with their effectiveness in MDCK cell homogenates as GlcCer synthase inhibitors. Some differences can be expected due to differences in sensitivity of the synthase occurring in each cell type (the synthases were assayed only in MDCK cells).

Growth inhibition by each of the most active BML compounds occurred in an unusually small range of concentrations (e.g., the slopes of the cytotoxic regions are unusually steep). Similar rapid drop-offs were seen in another series of tests with 9 L cells, in which BML-119 yielded 71% of the control growth with 1 μM inhibitor, but only 3% of control growth with 3 μM. Growth was 93% of control growth with 2 μM BML-130 but only 5% of controls with 3 μM inhibitor. While some clinically useful drugs also show a narrow range of effective concentrations, this is a relatively uncommon relationship.

When the erythro- and threo-stereoisomeric forms of BML-119 (-129 and -130) were compared, they were found to have similar effects on tumor cell growth (Table 4). This observation is similar to the results with PDMP isomers in fibroblasts cited above (Uemura, K. et al., "Effect of an Inhibitor of Glucosylceramide Synthesis on Cultured Rabbit Skin Fibroblasts," *J. Biochem.*, (Tokyo) 108:525-530 (1990)). Since enzymes are optically active and since stereoisomers and enantiomers of drugs can differ greatly in their effect on enzymes, it is likely that BML-129 and BML-130 work on different sites of closely related metabolic steps.

FIG. **2** shows the amount of cellular protein per dish for MDCK cells cultured for 24 h in medium containing different concentrations of the separated erythro- and threo-isomers of BML-119, as percent of the incorporation by cells in standard medium. Each point shown in FIG. **2** is the average of values from three plates, with error bars corresponding to one standard deviation.

FIG. **3** shows [^{3}H]thymidine incorporation into DNA of MDCK cells incubated as in FIG. **2**. The values in FIG. **3** are normalized on the basis of the protein content of the incubation dishes and compared to the incorporation by cells in standard medium.

FIGS. **2** and **3** thus provide comparison of the two stereoisomers with MDCK cells. The isomers were found to inhibit growth and DNA synthesis with similar effectiveness. Thus, the MDCK cells behaved like the human tumor cells with regard to IC_{50} and the narrow range of concentrations resulting in inhibition of protein and DNA synthesis.

Surprisingly, the aliphatic inhibitor IV-231B exerted no inhibitory effect on MDCK cell growth when incubated at 20 μM for 1 day or 1 μM for 3 days. Tests with a longer growth period, 5 days, in 5 μM inhibitor also showed no slowing of growth. The dishes of control cells, which contained BSA as the only additive to the medium, contained 3.31±0.19 mg of protein, while the IV-231B/BSA treated cells contained 3.30±0.04 mg.

Lipid changes induced in the cells. Examination by TLC of the alkali-stable MDCK lipids after a 24 h incubation disclosed that BML-130 was more effective than BML-129 in lowering GlcCer levels, as expected from its greater effectiveness in vitro as a glucosyltransferase inhibitor. The level of GlcCer, estimated visually, was greatly lowered by 0.3 μM BML-130 or 0.5 μM BML-129. The levels of the other lipids visible on the plate (mainly sphingomyelin (SM), cholesterol, and fatty acids) were changed little or not at all. BML-129 and the GlcCer synthase inhibitor, BML-130, were readily detected by TLC at the various levels used, showing that they were taken up by the cells during the incubation period at dose-dependent rates. Lactosylceramide overlapped the inhibitor bands with solvent D but was well separated with solvent E, which brought the inhibitors well above lactosylceramide.

Ceramide accumulation was similar for both stereoisomers (data not shown). An unexpected finding is that noticeable ceramide accumulation appeared only at inhibitor concentrations that were more than enough to bring GlcCer levels to a very low point (e.g., at 2 or 4 μM). The changes in ceramide concentration were quantitated in a separate experiment by the diglyceride kinase method, which allows one to also determine diacylglycerol (DAG) concentration (Preiss, J. E. et al., "Quantitative Measurement of SN-1,2-Diacylglycerols Present in Platelets, Hepatocytes, and Ras- and Sis-Transformed Normal Rat Kidney Cells," *J. Biol. Chem.*, 261:8597-8600 (1986)). The results (Table 5) are similar to the visually estimated ones: at 0.4 μM BML-129 or -130 there was little effect on ceramide content but at 4 μM inhibitor, a substantial increase was observed. (While the duplicate protein contents per incubation dish were somewhat erratic in the high-dose dishes, in which growth was slow, the changes were nevertheless large and clear.) Accumulation of ceramide had previously been observed with PDMP, at a somewhat higher level of inhibitor in the medium (Shayman, J. A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride,"

J. Biol. Chem., 266:22968-22974 (1991)). From the data for cellular protein per incubation dish, it can be seen that there was no growth inhibition at the 0.4 μM level with either compound but substantial inhibition at the 4 μM level, especially with the glucosyltransferase inhibitor, BML-130. This finding is similar to the ones made in longer incubations with human cancer cells.

TABLE 5

Effects of BML-129 and -130 on MDCK Cell Growth and the Content of Ceramide and Diacylglycerol

Growth Medium	Protein μg/dish	Ceramide nmol/mg	Diglyceride protein
Controls	490	1.04	4.52
	560	0.96	5.61
0.4 μm BML-129	500	1.29	5.51
	538	0.99	5.13
0.4 μm BML-130	544	0.94	4.73
	538	0.87	5.65
4 μm BML-129	396	3.57	9.30
	311	3.78	9.68
4 μm BML-130	160	5.41	11.9
	268	3.34	8.71

In a separate study of ceramide levels in MDCK cells, BML-130 at various concentrations was incubated with the cells for 24 h. The ceramide concentration, measured by TLC densitometry, was 1.0 nmol/mg protein at 0.5 μM, 1.1 ar 1 μM, 1.5 at 2 μM, and 3.3 at 4 μM. The results with BML-129 were virtually identical.

It is interesting that the accumulation of ceramide paralleled an accumulation of diacylglycerol (DAG), as observed before with PDMP (Shayman, J. A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.*, 266:22968-22974 (1991)). DAG is ordinarily considered to be an activator of protein kinase C and thus a growth stimulator, but the low level of GlcCer in the inhibited cells may counteract the stimulatory effect. Ceramide reacts with lecithin to form SM and DAG, so it is possible that the increased level of the latter reflects enhanced synthesis of the phosphosphingolipid rather than an elevated attack on lecithin by phospholipase D. Arabinofuranosylcytosine (ara-C), an antitumor agent, also produces an elevation in the DAG and ceramide of HL-60 cells (Strum, J. C. et al., "1-β-D-Arabinofuranosylcytosine Stimulates Ceramide and Diglyceride Formation in HL-60 Cells," *J. Biol. Chem.*, 269:15493-15497 (1994)).

TLC of MDCK cells grown in the presence of 0.02 to 1 μM IV-231B for 3 days showed that the inhibitor indeed penetrated the cells and that there was a great depletion of GlcCer, but no ceramide accumulation. The depletion of GlcCer was evident even at the 0.1 μM level and virtually no GlcCer was visible at the 1 μM level; however, the more polar GSLs were not affected as strongly. After incubation for 5 days in 5 μM inhibitor, all the GSLs were virtually undetectable. The ceramide concentrations in the control and depleted cells were very similar: 13.5±1.4 vs 13.9±0.2 μg/mg protein.

The lack of ceramide accumulation in cells exposed to the aliphatic inhibitors was examined further to see if it might be due to differential actions of the different inhibitors on additional enzymes involving ceramide metabolism. For example, IV-231B might block ceramide synthase and thus prevent accumulation despite the inability of the cells to utilize ceramide for GlcCer synthesis. However, assay of ceramide synthase in homogenized cells showed it was not significantly affected by 5 μM inhibitors (Table 6). There did appear to be moderate inhibition at the 50 μM level with PDMP and the aliphatic inhibitor.

TABLE 6

Effect of Inhibitors on Acid and Neutral Ceramidases and Ceramide Synthase of MDCK Cells

Inhibitor Tested	Enzyme Activity (% of control) Ceramidase pH 4.5	Ceramidase pH 7.4	Ceramide Synthase
D-threo-PDMP, 5 μM	97 ± 4	116 ± 19	99 ± 5
D-threo-PDMP, 50 μM	133 ± 13[a]	105 ± 11	66 ± 9[a]
BML-129, 5 μM	108 ± 8	100 ± 0	97 ± 0
BML-129, 50 μM	171 ± 26[a]	99 ± 2	102 ± 1
BML-130, 5 μm	107 ± 11	100 ± 15	108 ± 10
BML-130, 50 μm	160 ± 21[a]	100 ± 15	106 ± 29
IV-231B, 5 μm	106 ± 3	116 ± 20	90 ± 8
IV-231B, 50 μm	113 ± 8	112 ± 3	71 ± 18[a]

[a]Notable differences.

Assay of the two kinds of ceramidase (Table 6) showed that there was no effect of either the aliphatic or aromatic inhibitors at the 5 μM level, at which point cell growth is completely stopped in the case of the pyrrolidino compounds. At the 50 μM level, however, the acid enzyme was stimulated markedly by the aromatic inhibitors, particularly the two stereoisomeric forms of the pyrrolidino compound.

Sphingomyelin synthase was unaffected by PDMP or the aliphatic inhibitor but BML-129 and -130 produced appreciable inhibition at 50 μM (54% and 61%, respectively) (Table 7).

TABLE 7

Effect of Inhibitors on Acid and Neutral Sphingomyelinases and Sphingomyelin Synthase

Inhibitor Tested	Enzyme Activity (% of control) Sphingomyelinase pH 4.5	Sphingomyelinase pH 7.1	Sphingomyelinase Synthase[a]
D-threo-PDMP, 5 μM	102 ± 3	121 ± 13	
D-threo-PDMP, 50 μM	100 ± 3	108 ± 8	
BML-129, 5 μM	108 ± 4	105 ± 11	84 ± 27
BML-129, 50 μM	97 ± 3	142 ± 11[b]	46 ± 11[b]
BML-130, 5 μM	109 ± 1	110 ± 7	87 ± 14
BML-130, 50 μM	114 ± 2	152 ± 14	39 ± 18[b]
IV-231B, 5 μM	101 ± 7	131 ± 3[b]	
IV-231B, 50 μM	112 ± 11	120 ± 3[b]	

[a]Data for PDMP and IV-231B are not shown here as they were tested in other experiments; no effect was seen.
[b]Notable differences.

Neutral sphingomyelinase (SMase) was distinctly stimulated by the aliphatic inhibitor, IV-231B, even at 5 μM (Table 7). From this one would expect that the inhibitor would produce accumulation of ceramide, yet it did not. The two pyrrolidino compounds produced appreciable stimulation at the 50 μM level. No significant effects were obtained with acid SMase.

Discussion

The present invention shows that the nature and size of the tertiary amine on ceramide-like compounds exerts a strong influence on GlcCer synthase inhibition, a 5-membered ring being most active. It also shows that the phenyl ring used

previously to simulate the trans-alkenyl chain corresponding to that of sphingosine could, with benefit, be replaced with the natural alkenyl chain.

Findings with the most active GlcCer synthase inhibitors in growth tests compare favorably with evaluations of some clinically useful chemotherapeutic agents on three of the tumor cell lines in the same Drug Evaluation Core Laboratory. The IC_{50} values were 0.2 to 6 μM for cisplatin, 0.02 to 44 μM for carboplatin, 0.03 to 0.2 μM for methotrexate, 0.07 to 0.2 μM for fluorouracil, and 0.1 to 1 μM for etoposide. Unlike these agents, the compounds of the present invention yielded rather similar effects with all the cell types, including MDCK cells, and thus have wider potential chemotherapeutic utility. This uniformity of action is consistent with the idea that GSLs play a wide and consistent role in cell growth and differentiation.

An important observation from the MDCK cell study is that strong inhibition of cell growth and DNA synthesis occurred only at the same concentrations of aromatic inhibitor that produced marked ceramide accumulation. This observation supports the assertion that ceramide inhibits growth and enhances differentiation or cell death (Bielawska, A. et al., "Modulation of Cell Growth and Differentiation by Ceramide," *FEBS Letters,* 307:211-214 (1992)). It also agrees with previous work with octanoyl sphingosine, a short chain ceramide that produced greatly elevated levels of natural ceramide and slowed growth (Abe, A. et al., "Metabolic Effects of Short-Chain Ceramide and Glucosylceramide on Sphingolipids and Protein Kinase C," *Eur. J. Biochem.,* 210:765-773 (1992)). It is also in agreement with a finding that some synthetic, nonionic ceramide-like compounds did not inhibit GlcCer synthase even though they behave like ceramide in blocking growth (Bielawska, A. et al., "Ceramide-Mediated Biology. Determination of Structural and Stereospecific Requirements Through the Use of N-Acyl-Phenylaminoalcohol Analogs," *J. Biol. Chem,* 267: 18493-18497 (1992)). Compounds tested included 20 μM D-erythro-N-myristoyl-2-amino-1-phenyl-1-propanol, its L-enantiomer, the four stereoisomers of N-acetylsphinganine, and N-acetylsphingosine. Furthermore, the lack of growth inhibition and ceramide accumulation in cells treated with the aliphatic inhibitor IV-231B is also consistent with the correlation between ceramide level and growth rate.

The accumulation of ceramide that occurred at higher levels of GlcCer synthase inhibitors could be attributed not only to blockage of ceramide utilization, but also to blockage of SM synthesis or ceramide hydrolase. This possibility is especially relevant to the R,S-, S,R-, and S,S-isomers, which seem to exert effects on sphingolipids without strongly inhibiting GlcCer synthesis. The tests with both the DL-erythro-pyrrolidino inhibitor (BML-129) and the DL-threo-pyrrolidino inhibitor (BML-130), at a level producing strong growth inhibition, showed that neither material at a low concentration inhibited the enzymes tested in vitro (Tables 6 and 7) but they did cause growth inhibition as well as accumulation of ceramide. PDMP, at relatively high concentrations (50 μM), was found to inhibit SM synthase in growing CHO cells (Rosenwald, A. G. et al., "Effects of a Sphingolipid Synthesis Inhibitor on Membrane Transport Through the Secretory Pathway," *Biochemistry,* 31:3581-3590 (1992)). In the test with MDCK homogenates, it did not inhibit this synthase, in agreement with the finding that labeled palmitate incorporation into SM was stimulated by PDMP (Shayman, J. A. et al., "Modulation of Renal Epithelial Cell Growth by Glucosylceramide: Association with Protein Kinase C, Sphingosine, and Diacylglyceride," *J. Biol. Chem.,* 266:22968-22974 (1991)).

Retinoic acid is a growth inhibitor of interest in cancer chemotherapy and a possible adjunct in the use of the inhibitors of the present invention. It has been found to elevate ceramide and DAG levels (Kalen, A. et al., "Elevated Ceramide Levels in GH4C1 Cells Treated with Retinoic Acid," *Biochim. Biophys. Acta,* 1125:90-96 (1992)) and possibly lower lecithin content (Tang, W. et al., "Phorbol Ester Inhibits 13-Cis-Retinoic Acid-induced Hydrolysis of Phosphatidylinositol 4,5-Bisphosphate in Cultured Murine Keratinocytes: a Possible Negative Feedback Via Protein Kinase C-Activation," *Cell Bioch. Funct.,* 9:183-191 (1991)).

D-threo-PDMP was found to be rather active in delaying tumor cell growth or in producing complete cures in mice (Inokuchi, J. et al., "Antitumor Activity in Mice of an Inhibitor of Glycosphingolipid Biosynthesis," *Cancer Lett,* 38:23-30 (1987)) but high doses were needed. From the data in FIG. **1**, the inhibitors of the present invention are approximately 30 times as active, so the dosage levels are typical of clinically useful drugs. The need to use high doses with PDMP was attributed to rapid inactivation by cytochrome P450 (Shukla, A. et al., "Metabolism of D-[^{3}H]PDMP, an Inhibitor of Glucosylceramide Synthesis, and the Synergistic Action of an Inhibitor of Microsomal Monooxygenase," *J. Lipid Res.,* 32:713-722 (1991)). Cytochrome P450 can be readily blocked by various nontoxic drugs such as cimetidine, therefore high levels of the compounds of the present invention can be maintained.

SPECIFIC EXAMPLE 2

A series of inhibitors based on substitutions in the phenyl ring of P4 were synthesized and studied. It was found that the potency of the inhibitors in blocking GlcCer synthase was mainly dependent upon hydrophobic and electronic properties of the substituent. Surprisingly, a linear relationship was found between log [IC_{50}] and hydrophobic parameter (π)+electronic parameter (δ). This correlation suggested that electron donating and hydrophilic characters of the substituent enhance the potency as an inhibitor. This observation resulted in the synthesis of novel compounds that are more active in blocking glucosylceramide formation. Two compounds, dioxy D-t-P4 compounds, D-t-3',4'-ethylenedioxy-P4 and D-t-4'-hydroxy-P4, were observed to be significantly more potent than other tested inhibitors. In particular, at 11.3 nM D-t-3',4'-ethylenedioxy-P4, 80% of glucosylceramide in MDCK cell was depleted without any ceramide accumulation and cell growth inhibition. The potency of D-t-3',4'-ethylenedioxy-P4 appears to be not only regulated by hydrophobic and electronic properties but also by stearic properties of the substituents on the phenyl group.

Materials and Methods

Materials. The acetophenones and amines were from Aldrich Chemical Co., St. Louis, Mo., Lancaster Synthesis Inc., Windham, N.H. and Maybridge Chemical Co., Cornwall, UK. Silica gel for column chromatography (70-230 mesh ASTM) and Silica gel thin layer chromatography plates were purchased from Merck Co. The reagents and their sources were: non-hydroxy fatty acid ceramide from bovine brain and delipidated bovine serum albumin (BSA) from Sigma; dioleoyphosphatidylcholine from Avanti; DL-dithiothreitol from Calbiochem; 1-[^{3}H]-glucose uridine diphosphate from NEN. Octanoylsphingosine, glucosylceramide and sodium sulfatide were prepared as previously described. Abe, A. et al., *Eur. J. Biochemistry,* 210:765-773 (1992).

General synthesis of inhibitors. The aromatic inhibitors were synthesized by the Mannich reaction from 2-N-acylaminoacetophenone, paraformaldehyde, and pyrrolidine, and then the reduction from sodium borohydride as described before. Inokuchi, J. et al., *J. Lipid. Res.,* 28:565-571 (1987); Abe, A. et al., *J. Lipid. Res.,* 36:611-621 (1995). The reaction produces a mixture of four isomers, due to the presence of two asymmetric centers. For these syntheses in which phenyl-substituted starting materials were used, the chloro, methoxy, methylenedioxy, methyl groups in the acetophenone structure were brominated and converted to the primary amine. Bromation of the methoxyacetophenone, dimethyoxyacetophenone, 3',4'-(methylenedioxy)acetophenone were performed in chloroform at room temperature and recrystallized from ethyl acetate and hexane.

Synthesis of 1-(4'-hydroxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol. The synthesis of 1-(4'-hydroxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol is described in detail in FIG. **8**. This synthesis differs from those of the other compounds because of the need for the placement of a protecting group on the free hydroxyl (step 1) and its subsequent removal (step 7). All other syntheses employ a similar synthetic scheme (steps 2 to 6).

4'-Benzyloxyacetophenone formation (step 1) 4'-Hydroxyacetophenone (13.62 g, 100 mmol), benzylbromide (17.1 g, 100 mmol), and cesium carbonate (35.83 g, 100 mmol) were added to tetrahydrofuran at room temperature and stirred overnight. The product was concentrated to dryness and recrystallized from ether and hexane to yield 15 g of 4'-benzyloxyacetophenone which appeared as a white powder. An R_f of 0.42 was observed when resolved by thin layer chromatography using methylene chloride. ^{1}H nmr (δ, ppm, $CDCl_3$), 7.94 (2H, δ, 8.8 Hz, O—Ar—C(O)), 7.42 (5H, m, Ar'CH_2O—), 7.01 (2H, δ, 8.8 Hz, O—Ar—C(O)), 5.14 (2H, s, Ar'CH_2O—), 2.56 (3H, S, CH_3).

Bromination of 4'-benzyloxyacetophenone (step 2) Bromine (80 mmol) was added dropwise over 5 min to a stirred solution of 4'-benzyloxyacetophenone (70 mmol) in 40 ml chloroform. This mixture was stirred for an additional 5 min and quenched with saturated sodium bicarbonate in water until the pH reached 7. The organic layers were combined, dried over $MgSO_4$, and concentrated to dryness. The crude mixture was purified over a silica gel column and eluted with methylene chloride to yield 2-bromo-4'-benyloxyacetophenone. An R_f of 0.62 was observed when resolved by thin layer chromatography using methylene chloride. ^{1}H nmr (δ, ppm, $CDCl_3$), 7.97 (2H, δ, 9.2 Hz, O—Ar—(O)), 7.43 (5H, m, Ar'CH_2O—), 7.04 (2H, δ, 9.0 Hz, O—Ar—C(O)), 5.15 (2H, s, Ar'CH_2O—), 4.40 (2H, s, CH_2Br).

2-Amino-4'-benzyloxyacetophenone HCl formation (step 3) Hexamethylenetetramine (methenamine, 3.8 g, 23 mmol) was added to a stirred solution of 2-bromine-4'-benyloxyacetophenone (6.8 g, 23 mmol) in 100 ml chloroform. After 4 h the crystalline adduct was filtered and washed with chloroform. The product was dried and heated with 150 ml methanol and 8 ml of concentrated HCl in an oil bath at 85° C. for 3 h. Upon cooling the precipitated hydrochloride salt (2.5 g) was removed by filtration. The filtrate was left at −20° C. overnight and additional product (2.1 g) was isolated. The yield was 4.6 g (82.6%). $[M^+H]^+$: 242 for $C_{15}H_{16}NO_2$. ^{1}H nmr (δ, ppm, $CDCl_3$), 8.38 (2H, bs, NH_2), 7.97 (2H, δ, 8.8 Hz, O—Ar—C(O)), 7.41 (5H, m, Ar'CH2O—), 7.15 (2H, δ, 8.6 Hz, OArC(O)), 5.23 (2H, s, Ar'CH_2O), 4.49 (2H, s, CH_2NH_2).

2-Palmitoylamino-4'-benyloxyacetophenone formation (step 4) Sodium acetate (50% in water, 29 ml) was added in three portions to a stirred solution of 2-amino-4'-benzyloxyacetophenone HCl (4.6 g, 17 mmol) and tetrahydrofuran (200 ml). Palmitoyl chloride (19 mmol) in tetrahydrofuran (25 ml) was added dropwise over 20 min yielding a dark brown solution. The mixture was stirred overnight at room temperature. The aqueous fraction was removed by use of a separatory funnel and chloroform/methanol (2/1, 150 ml) was added to the organic layer which was then washed with water (50 ml). The yellow aqueous layer was extracted once with chloroform (50 ml). The organic solutions were then pooled and rotoevaporated until near dryness. The residue was redissolved in chloroform (100 ml) and crystallized by the addition of hexane (400 ml). The flask was cooled to 4° C. for 2 h. The crystals were filtered and washed with cold hexane and dried in a fume hood overnight. The product yield was 3.79 g (8 mmol). An R_f of 0.21 was observed when resolved by thin layer chromatography using methylene chloride. $[M^+H]^+$: 479 for $C_{31}H_{45}NO_3$. ^{1}H mm (δ, ppm, $CDCl_3$), 7.96 (2H, δ, 8.8 Hz, O—Ar—C(O)), 7.40 (5H, m, Ar'CH_2O—), 7.03 (2H, δ, 8.8 Hz, O—Ar—C(O)), 6.57 (1H, bs, NH_2), 5.14 (2H, s, Ar'CH_2O—), 4.71 (2H, s, C(O)CH_2NHC(O)), 2.29 (2H, t, 7.4 Hz, C(O)$CH_2(CH_2)_{13}CH_3$), 1.67 (2H, m, C(O)$CH_2(CH_2)_{13}CH_3$), 0.87 (3H, t, 6.7 Hz, C(O)$CH_2(CH_2)_{13}$ CH_3).

1-(4'-Benzyloxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol formation (steps 5 and 6) 2-Palmitoylamino-4'-benzyloxyacetophenone (3.79 g, 8.0 nmol), paraformaldehyde (0.25 g, 2.7 mmol), pyrrolidine (0.96 ml, 11.4 mmol) and ethanol (70 ml) were stirred under nitrogen. Concentrated HCl (0.26 ml) was added through the condensor and the mixture was heated to reflux for 16 h. The resultant brown solution was cooled on ice and then sodium borohydride (1.3 g, 34 mmol) was added in three portions. The mixture was stirred at room temperature overnight, and the product was dried in a solvent evaporator. The residue was redissolved in dichloromethane (130 ml) and hydrolyzed with 3N HCl (pH~4). The aqueous layer was extracted twice with dichloromethane (50 ml). The organic layers were pooled and washed twice with water (30 ml), twice with saturated sodium chloride (30 ml), and dried over anhydrous magnesium sulfate. The dichloromethane solution was rotoevaporated to a semisolid and purified by use of a silica rotor using a solvent consisting of 10% methanol in dichloromethane. This yielded a mixture of DL-threo- and DL-erythro enantiomers (2.53 g, 4.2 mmol). An R_f of 0.43 for the erythro diastereomers and 0.36 for the threo diastereomers was observed when resolved by thin layer chromatography using methanol:methylene chloride (1:9). $[M^+H]^+$: 565 for $C_{36}H_{56}N_2O_3$.

1-(4'-Hydroxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol formation (step 7): A suspension of 20% Pd/C (40 mg) in acetic acid (15 ml) was stirred at room temperature under a hydrogen balloon for 15 min. 1-(4'-Benzyloxy)phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (420 mg, 0.74 mmol) was added and the solution was stirred overnight. The suspension was filtered through a glass frit, and the filter was rinsed with acetic acid:methylene chloride (1:1, 5 ml). The filtrate was concentrated in vacuo and crystallized to yield a pale yellow semisolid (190 mg, 0.4 mmol). An R_f of 0.21 was observed when resolved by thin layer chromatography using methanol:methylene chloride (1:9). $[M^+H]^+$: 475 for $C_{29}H_{50}N_2O_3$. ^{1}H nmr (δ, ppm, $CDCl_3$), 7.13 (4H, m, ArCHOH—), 7.14 (1H, δ, 6.9 Hz, —NH—), 5.03 (1H, δ, 3.3 Hz, CHOH—), 4.43 (1H, m, c-$(CH_2CH_2)_2NCH_2CH$), 3.76 (2H, m, c-$(CH_2CH_2)_2$N—), 3.51 (1H, m, c-$(CH_2CH_2)_2NCH_2$—), 3.29 (1H, m, c-$(CH_2CH_2)_2NCH_2$—), 2.97 (3H, m, c-$(CH_2CH_2)_2$N— and ArC(OH)H—), 2.08 (6H, m, —C(O)$CH_2(CH_2)_{13}CH_3$ and

c-$(CH_2CH_2)_2N$—, 1.40 (2H, m, $C(O)CH_2CH_2(CH_2)_{12}CH_3$), 1.25 (2H, m, —$C(O)CH_2CH_2(CH_2)_{12}CH_3$), 0.87 (3H, t, 6.7 Hz, $C(O)CH_2(CH_2)_{13}CH_3$).

Synthesis of D-threo-1-(3',4'-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol

2-Amino-3',4'-(ethylenedioxy)acetophenone HCl: Hexamethylenetetramine (methenamine, 5.4 g, 0.039 mol) was added to a stirred solution of phenacylbromide (10.0 g, 0.039 mol) in 200 ml chloroform. After 2 h, the crystalline adduct was filtered and washed with chloroform. The product was then dried and heated with methanol (200 ml) and concentrated HCl (14 ml) in an oil bath at 85° C. for 2 h. On cooling, the precipitated ammonium chloride was removed by filtration and the filtrate was left in a freezer overnight. After filtration the crystallized phenacylamine HCl was washed with cold isopropanol and then with ether. The yield of this product was ~7.1 g (81%).

2-Palmitoylamino-3',4'-(ethylenedioxy)acetophenone: Aminoacetophenone HCl (7.1 g, 31 mmol) and tetrahydrofuran (300 ml) were placed in a 1 liter three-neck round bottom flask with a large stir bar. Sodium acetate (50% in water, 31 ml) was added in three portions to this suspension. Palmitoyl chloride (31 ml, 10% excess, 0.036 mol) in tetrahydrofuran. (25 ml) was then added dropwise over 20 min to yield a dark brown solution. This mixture was then stirred for an additional 2 h at room temperature. The resultant mixture was poured into a separatory funnel to remove the aqueous solution. Chloroform/methanol (2/1, 150 ml) was then added to the organic layer and washed with water (50 ml). The yellow aqueous layer was extracted once with chloroform (50 ml). The organic solutions were pooled and rotoevaportated until almost dry. The residue was redissolved in chloroform (100 ml) and crystallized by the addition of hexane (400 ml). The flask was then cooled to 4° C. for 2 h. The crystals were filtered and washed with cold hexane until they were almost white and then dried in a fume hood overnight. The yield of the product was 27 mmol (1.6 g).

D-threo-1-(3',4'-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol: Almitoylaminoacetophenone (11.6 g, 0.027 mol), paraformaldehyde (0.81 g, 0.009 mol), pyrrolidine (3.6 ml, 0.042 mmol) and ethanol (250 ml) were added to a 500 ml round flask under nitrogen flow. Concentrated HCl (0.8 ml) was added to this mixture through the reflux condenser and the mixture was refluxed for 16 h. The brown solution was cooled in an ice-bath. Sodium borohydride (2.28 g, 0.06 mol) was added in three portions. This mixture was stirred at room temperature for 3 h and then rotoevaporated. The residue was dissolved in 130 ml of dichloromethane and the borate complex hydrolyzed with HCl (3N) until the pH was approximately 4. The aqueous layer was extracted twice with 50 ml dichloromethane. The organic layers were pooled and washed twice with H_2O (30 ml), saturated NaCl (30 ml) and dried over anhydrous $MgSO_4$. The dichloromethane solution was rotoevaporated to a viscous oil which was purified by use of a Chromatotron with a solvent consisting of 10% methanol in dichloromethane to obtain a mixture of DL-threo and erythro enantiomers (2.24 g, 0.004 mol).

Resolution of inhibitor enantiomers. High performance liquid chromatography (HPLC) resolution of the enantiomers of DL-threo and DL-erythro are performed using a preparative HPLC column (Chirex 3014: [(S)-val-(R)-1-a-naphtyl)ethylamine, 20×250 mm: Phenomenex], eluted with hexane-1,2-dichloroethane-ethanol-trifluroacetic acid 64:30:5.74:0.26, at a flow rate of 8 ml/min. The column eluent was monitored at 254 nm in both the preparative and analytical modes. Isolated products were reinjected until pure by analytical HPLC analysis, determined using an analytical Chirex 3014 column (4.6×250 mm) and the above solvent mixture at flow rate of 1 ml/min.

Glycosylceramide synthase activity. The enzyme activity was measured by the method previously described in Skukla, G. et al., *Biochim. Biophys. Acta,* 1083:101-108 (1991). MDCK cell homogenate (120 μg of protein) was incubated with uridinediphosphate [^{3}H]glucose (100,000 cpm) and liposomes consisting of 85 μg octanoylsphingosine, 570 μg dioleoyphosphatidylcholine and 100 μg sodium sulfatide in 200 μl of reaction mixture and kept for 1 h at 37° C. P4 and P4 derivatives dissolved in dimethyl sulfoxide were dispersed into the reaction mixture after adding liposomes. The final concentration of dimethyl sulfoxide was kept 1% under which the enzyme activity was not at all inhibited.

Cell culture and lipid extraction. One half million of MDCK cells were seeded into 10 cm style dish containing 8 ml serum free DMEM supplemented medium. Shayman, J. A. et al., *J. Biol. Chem.,* 265:12135-12138 (1990). After 24 h the medium was replaced with 8 ml of the medium containing 0, 11.8, 118 or 1180 nM D-t-P4, D-t-3',4'-ethylenedioxy-P4 or D-4'-hydroxy)-P4. The GlcCer synthase inhibitors were added into the medium as a one to one complex with delipidated BSA. Abe, A. et al., *J. Lipid. Res.,* 36:611-621 (1995); Abe, A. et al., *Biochim. Biophys. Acta,* 1299:331-341 (1996). The cells were incubated for 24 h or 48 h with the inhibitors. After the incubation, the cells were washed twice with 8 ml of cold PBS and fixed with 2 ml of cold methanol. The fixed cells were scraped and transferred to a glass tube. Another one ml of methanol was used to recover the remaining cells in the dish.

Three ml of chloroform was added to the tube and briefly sonicated using a water bath type sonicator. After centrifugation at 800 g for 5 min, the supernatant was transferred into another glass tube. The residues were reextracted with chloroform/methanol (1/1). After the centrifugation, the resultant supernatant was combined with the first one. The residues were air-dried and kept for protein analysis. Adding 0.9% NaCl to the supernatant combined, the ratio of chloroform/methanol/aqueous was adjusted to 1/1/1. After centrifugation 800 g for 5 min, the upper layer was discarded. Methanol/water (1/1) with the same amount of volume of the lower layer was used to wash. The resultant lower layer was transferred into a small glass tube and dried down under a stream of nitrogen gas. A part of the lipid was used for lipid phosphate determination. Ames, B. N., *Methods Enzymol.,* 8:115-118 (1966). The remainder was analyzed using HPTLC (Merck).

Results

Synthesis of P4 and P4 derivatives. The preparation of P4 derivatives utilized the Mannich reaction from 2-N-acylaminoacetophenone, paraformaldehyde, and pyrrolidine, and then the reduction of DL-pyrrodino ketone from sodium borohydride. In most cases, no isolation of DL-pyrrodino ketones were performed to maintain solubility. The overall yields of the DL-threo and DL-erythro syntheses were ~10-30%. These derivatives were purified by the either silica gel column or rotors with solvent 5-12% methanol in dichloromethane to optimize the separation from the chiral column. To obtain the best separation, each injection con-

tains no more than 150 mg, and fractions were pooled to obtain sufficient quantity of isomer of D-threo for further biological characterization.

Resolution of PDMP homologues by chiral chromatography. The structures of the parent compound, D-threo-P4 and the phenyl-substituted homologues including the new dioxy-substituted and 4'-hydroxy-P4 homologues are shown in FIG. **9**. Initially the effect of each P4 isomer separated by chiral chromatography on GlcCer synthase activity was determined (FIG. **10**). Four peaks were observed for the chiral separation of P4. Peaks 1 and 2 represented the erythro diastereomers and 3 and 4 represented the threo diastereomers as determined by a sequential separation of the P4 mixture by reverse phase chromatography followed by the chiral separation. The enzyme activity was specifically inhibited by the fourth peak, the D-threo isomer (FIG. **4**A). This specificity for the D-threo enantiomer was consistent with the previous results observed in PDMP and PDMP homologues (2-4). The IC_{50} of D-threo-P4 was 0.5 mM for GlcCer synthase activity measured in the MDCK cell homogenates.

Effects of P4 and P4 Derivatives with a Single Substituent of Phenyl Group on GlcCer Synthase Activity. The effect of each P4 isomer on GlcCer synthase activity was analyzed. The reaction was carried out in the absence or presence of 0.1, 1.0 or 10 μM P4 (FIG. **4**A) or p-methoxy-P4 (FIG. **4**B). As shown in FIG. **4**A, the enzyme activity was specifically inhibited by D-threo isomer. In FIG. **4**A, the symbols are denoted as follows: D-threo (○), D-erythro (□), L-threo and (•), L-erythro (Δ). This specificity is consistent with previous results observed in PDMP and PDMP homologs. Inokuchi, J. et al., *J. Lipid. Res.* 28:565-571 (1987); Abe, A. et al., *J. Lipid. Res.* 36:611-621 (1995). The IC_{50} of D-t-P4 was 500 nM.

As set forth herein, the addition of a p-methoxy group to DL-t-P4 was found to enhance the effect of the inhibitor on the enzyme activity. Abe, A. et al., *J. Lipid. Res.,* 36:611-621 (1995). As shown in FIG. **4**B, it was confirmed that the enzyme activity was potently inhibited by D-threo-p-methoxy-P4 whose IC_{50} was 200 nM. In FIG. **4**B, □ denotes a mixture of D-erythro and L-threo isomers contaminated with a small amount of the D-threo isomer. Chiral chromatography of the four p-methoxy-P4 enantiomers failed to completely resolve to baseline each enantiomer (FIG. **10**). A slight inhibition of the enzyme activity by p-methyoxy-P4 in a combined D-erythro and L-threo mixture (peaks 2 and 3, FIG. **10**) was observed; this was due to contamination of the D-threo isomer (peak 4, FIG. **10**) into these fractions.

A series of D-t-P4 derivatives containing a single substituent on the phenyl group were investigated. As shown in Table 8, the potency of the derivatives as inhibitors were inferior to that of D-t-P4 or p-methoxy-D-t-P4. In many drugs, the influence of an aromatic substituent on the biological activity has been known and predicted. Hogberg, T. et al., Theoretical and experimental methods in drug design applied on antipsychotic dopamine antagonists. Larsen, P. K., and Bundgaard, H., "Textbook of Drug Design and Development," pp. 55-91 (1991). Generally IC_{50} is described as the following equation:

$$\log(1/IC_{50})=a(\text{hydrophobic parameter}(\pi)+b(\text{electronic parameter}(\sigma)+c(\text{stearic parameter})+d(\text{other descriptor})+e$$

where a, b, c, d and e are the regression coefficients. Hogberg, T. et al., Theoretical and experimental methods in drug design applied on antipsychotic dopamine antagonists. Larsen, P. K., and Bundgaard, H., "Textbook of Drug Design and Development," pp. 55-91 (1991).

The hydrophobic effect, π, is described by the equation $\pi=\log P_X-\log P_H$ where P_X is the partition coefficient of the substituted derivative and P_H is that of the parent compound, measured as the distribution between octanol and water.

The electronic substituent parameter, σ, was originally developed by Hammett (Hammett, L. P., In Physical Organic Chemistry, McGraw-Hill, New York (1940)) and is expressed as $\sigma=\log K_X-\log K_H$, where K_X and K_H are the ionization constants for a para or meta substituted derivative and benzoic acid respectively. Positive σ values represent electron withdrawing properties and negative a values represent electron donating properties.

The potency of D-threo-P4 and P4 derivatives as an inhibitor is mainly dependent upon two factors, hydrophobic and electronic properties, of a substituent of phenyl group (Table 8). Surprisingly, a linear relationship was observed between log (IC_{50}) and π+σ (FIG. **5**). These findings suggest that the more negative the value of π+σ, the more potent is D-threo-P4 derivatives made as GlcCer synthase inhibitor.

The data in Table 8 indicate that the potency of D-t-P4 and P4 derivatives as an inhibitor is mainly dependent upon two properties, hydrophobic and electronic properties, of a substituent of the phenyl group. Surprisingly, a linear relationship was observed between log(IC_{50}) and π+σ (FIG. **5**). These findings suggest that the more negative the value of π+σ, the more potent the D-t-P4 derivative as a GlcCer synthase inhibitor.

TABLE 8

D-threo-P4 derivative	σ + π*	IC_{50} (μM)**
p-methoxy	−0.29	0.2
P-4	0.00	0.5
m-methoxy-P4	0.10	0.6
p-methyl-P4	0.39	2.3
p-chloro-P4	0.94	7.2

*These values were estimated from the Table in Hogberg, T. et al., Theoretical and experimental methods in drug design applied on antipsychotic dopamine antagonists. Larsen, P. K., and Bundgaard, H., "Textbook of Drug Design and Development," pp. 55-91 (1991),for methoxy, σ_m 0.12, σ_p = −0.27, π = −0.02; hydro, σ = 0, π = 0; methyl, σ_p = −0.17, π = 0.56; chloro, σ_p = 0.23, π = 0.71.
**These values were derived from FIGS. 4A and 4B. For other compounds the same analytical approach as shown in FIGS. 4A and 4B was carried out to obtain the IC_{50}.

The p-hydroxy-substituted homologue was a significantly better GlcCer synthase inhibitor. The strong association between π+σ and GlcCer synthase inhibition suggested that a still more potent inhibitor could be produced by increasing the electron donating and decreasing the lipophilic properties of the phenyl group substituent. A predictably negative π+σ value would be observed for the p-hydroxy homologue. This compound was synthesized and the D-threo enantiomer isolated by chiral chromatography. An IC_{50} of 90 nM for GlcCer synthase inhibition was observed (FIG. **11**), suggesting that the p-hydroxy homologue was twice as active as the p-methoxy compound. Moreover, the linear relationship between the log (IC_{50}) and π+σ was preserved (open circle, FIG. **4**).

Effects of 3',4'-dioxy-D-threo-P4 Derivatives on GlcCer Synthase Activity. The result in FIG. **5** suggested that an electron donating and hydrophilic substituent of phenyl group makes the GlcCer synthase inhibitor potent. To attain further improvement of the inhibitor, another series of P4

derivatives with methylenedioxy, ethylenedioxy and trimethyldioxy substitutions on the phenyl group were designed (FIG. **9**).

As shown in FIG. **6**, the enzyme activity was markedly inhibited by D-t-3',4'-ethylenedioxy-P4 whose IC_{50} was 100 nM. In FIG. **6**, □ denotes D-t-3',4'-methylenedioxy-P4, ○ denotes D-t-3',4'-ethylenedioxy-P4, Δ denotes D-t-3',4'-trimethylenedioxy-P4 and • denotes D-t-3',4'-dimethyoxy-P4. One the other hand, the IC_{50} s for D-t-3',4'-methylenedioxy-P4 and D-t-3',4'-trimethylenedioxy-P4 were about 500 and 600 nM, respectively. These results suggest that the potency of D-t-3',4'-ethylenedioxy-P4 is not only regulated by hydrophobic and electronic properties but also by other factors, most likely stearic properties, induced from the dioxy ring on the phenyl group.

Interestingly, D-t-3',4'-dimethoxy-P4 was inferior to these dioxy derivatives, even to D-t-P4 or m- or D-t-p-methoxy-P4, as an inhibitor (FIG. **6**). As the parameters, σ_m, σ_p and π, for methoxy substituent are 0.12, −0.27 and −0.02, respectively (Hogberg, T. et al., Theoretical and experimental methods in drug design applied on antipsychotic dopamine antagonists. Larsen, P. K., and Bundgaard, H., "Textbook of Drug Design and Development," pp. 55-91 (1991)), the value of $\pi+\sigma$ of D-t-dimethoxy P4 is presumed to be negative. Therefore, the dimethoxy-P4 is thought to deviate quite far from the correlation as observed in FIG. **5**. There may be a repulsion between two methoxy groups in the dimethoxy-P4 molecule that induces a stearic effect that was negligible in mono substituent D-t-P4 derivatives studied in FIG. **5**. GlcCer synthase is thought to possess a domain that interacts with D-t-PDMP and PDMP homologs and that modulates the enzyme activity. Inokuchi, J. et al., *J. Lipid. Res.,* 28:565-571 (1987); Abe, A. et al., *Biochim. Biophys. Acta,* 1299:331-341 (1996). The stearic effect generated by an additional methoxy group may affect the interaction between the enzyme and the inhibitor. As a result, the potency as an inhibitor is markedly changed.

Distinguishing Between Inhibition of GlcCer Synthase and 1-O-acylceramide Synthase Inhibition. Prior studies on PDMP and related homologues revealed that both the threo and erythro diastereomers were capable of increasing cell ceramide and inhibiting cell growth in spite of the observation that only the D-threo enantiomers blocked GlcCer synthase. An alternative pathway for ceramide metabolism was subsequently identified, the acylation of ceramide at the 1-hydroxyl position, which was blocked by both threo and erythro diastereomers of PDMP. The specificities of D-threo-P4, D-threo-3',4'-ethylenedioxy-P4, and D-threo-(4'-hydroxy)-P4 for GlcCer synthase were studied by assaying the transacylase. Although there was an ca. 100 fold difference in activity between D-threo-3',4'-ethylenedioxy-P4, D-threo-(4'-hydroxy)-P4, and D-threo-P4 (IC_{50} 0.1 mM versus 10 mM) in inhibiting GlcCer synthase, the D-threo enantiomers of all three compounds demonstrated comparable activity in blocking 1-O-acylceramide synthase (FIG. **12**).

In order to determine whether inhibition of 1-O-acylceramide synthase was the basis for inhibitor mediated ceramide accumulation, the ceramide and diradylglycerol levels of MDCK cells treated D-threo-P4, D-threo-3',4'-ethylenedioxy-P4, and D-threo-(4'-hydroxy)-P4 were measured (Table 9). MDCK cells (5×10.sup.5) were seeded into a 10 cm dish and incubated for 24 h. Following the incubation, the cells were treated for 24 or 48 h with or without P4 or the phenyl substitute homologues. Both ceramide and diradylglycerol contents were determined by the method of Preis, J. et al., *J. Biol. Chem.,* 261:8597-8600 (1986). GlcCer content was measured densitometrically by a video camera and use of NIH image 1.49. Significant increases in both ceramide and diradylglycerol occurred only in cells treated with inhibitor concentrations in excess of 1 mM. This was approximately 30-fold lower than the concentration required for inhibition of the 1-O-acylceramide synthase assayed in the cellular homogenates. This disparity in concentration effects most likely reflects the ability of the more potent homologues to accumulate within intact cells. Abe, A. et al., *Biochim. Biophys. Acta,* 1299:331-341 (1996).

TABLE 9

GlcCer, ceramide and diradylglycerol content of MDCK cells treated with D-threo-P4, D-threo-3',4'-ethylenedioxy-P4, and D-threo-(4'-hydroxy)-P4

	Condition	Ceramide (pmol/nmol phospholipids)	Diradylglycerol (pmol/nmol phospholipids)
Control	24 h	4.53 ± 0.12	24.2 ± 2.36
	48 h	6.68 ± 0.49	32.3 ± 3.11
D-threo-P4	11.3 nM		
	24 h	5.33 ± 0.41*	24.1 ± 1.66
	48 h	5.68 ± 0.27*	29.6 ± 0.73
	113 nM		
	24 h	4.64 ± 0.38	26.6 ± 1.56
	48 h	7.08 ± 0.29	33.0 ± 2.63
	1130 nM		
	24 h	5.10 ± 0.35	27.1 ± 0.67
	48 h	9.74 ± 0.53	38.8 ± 1.11
D-threo-4'-hydroxy-P4	11.3 nM		
	24 h	4.29 ± 0.71	30.9 ± 2.01*
	48 h	6.70 ± 0.29	38.4 ± 1.44*
	113 nM		
	24 h	5.09 ± 0.95	31.5 ± 3.84*
	48 h	7.47 ± 0.29	41.5 ± 0.66*
	1130 nM		
	24 h	7.38 ± 0.13	38.5 ± 3.84*
	48 h	13.4 ± 1.03*	47.2 ± 2.51*
D-threo-3',4'-ethylenedioxy-P4	11.3 nM		
	24 h	5.24	22.0
		5.04	24.7
	113 nM		
	24 h	5.21	32.5
		5.21	41.6
	1130 nM		
	24 h	9.64	32.5
		13.0	41.6

*Denotes $p < 0.05$ by the Student t test. For the D-threo-(ethylenedioxy)-P4 only two determinations were made.

Effects of D-threo-P4, D-threo-4'-hydroxy-P4 and D-threo-3,4'-ethylenedioxy-P4 on GlcCer Synthesis and Cell Growth. To confirm the cellular specificity of D-threo-3',4'-ethylenedioxy-P4 and D-threo-(4'-hydroxy)-P4 as compared to D-threo-P4, MDCK cells were treated with different concentrations of the inhibitors. The total protein amount in each sample was determined by the BCA method. In GlcCer analysis, lipid samples and standard lipids were applied to the same HPTLC plate pre-treated with borate and developed in a solvent consisting of C/M/W (63/24/4). The level of GlcCer was estimated from a standard curve obtained using a computerized image scanner. The values were normalized on the basis of the phospholipid content. The results are shown in FIG. **7**, wherein each bar is the average values from three dishes, with error bars corresponding to one standard deviation. In the control, the total protein and GlcCer were 414±47.4 μg/dish and 24.3±1.97 ng/nmol phosphate, respectively.

Approximately 66 and 78% of the GlcCer was lost from the cells treated by 11.3 nM D-threo-4'-hydroxy-P4 and

D-threo-3',4'-ethylenedioxy-P4 respectively (FIGS. **7**, **14** and **15**). By contrast, only 27 percent depletion of GlcCer occurred in cells exposed to D-threo-P4 (FIG. **13**). A low level of GlcCer persisted in the cells treated with 113 or 1130 nM of either compound. This may be due to the contribution, by degradation, of more highly glycosylated sphingolipids or the existence of another GlcCer synthase that is insensitive to the inhibitor.

On the other hand, there was little difference in the total protein content between untreated and treated cells with 11.3 or 113 nM D-threo-4'-hydroxy-P4 and D-threo-3',4'-ethylenedioxy-P4 (FIGS. **14** and **15**). A significant decrease in total protein was observed in the cells treated with 1130 nM of either P4 homologue. In addition, the level of ceramide in the cells treated with 1130 nM D-threo-3',4'-ethylenedioxy-P4 and D-threo-(4'-hydroxy)-P4 was two times higher than that measured in the untreated cells (Table 9). There was no change in ceramide or diradylglycerol levels in cells treated with 11.3 nM or 113 nM concentrations of either compound. Similar patterns for GlcCer levels and protein content were observed at 48 h incubations.

The phospholipid content was unaffected at the lower concentrations of either D-threo-3',4'-ethylenedioxy-P4 or D-threo-(4'-hydroxy)-P4. The ratios of cell protein to cellular phospholipid phosphate (mg protein/nmol phosphate) were 4.94±0.30, 5.05±0.21, 4.84±0.90, and 3.97±0.29 for 0, 11.3, 113, and 1130 nM D-threo-3',4'-ethylenedioxy-P4 respectively, and 4.52±0.39, 4.35±0.10, and 3.68±0.99 for 11.3, 113, and 1130 nM D-threo-4'-hydroxy-P4 suggesting that the changes in GlcCer content were truly related to inhibition of GlcCer synthase activity. These results strongly indicate that the inhibitors D-threo-4'-hydroxy-P4 and D-threo-3',4'-ethylenedioxy-P4, are able to potently and specifically inhibit GlcCer synthesis in intact cells at low nanomolar concentrations without any inhibition of cell growth.

SPECIFIC EXAMPLE 3

Compositions within the scope of invention include those comprising a compound of the present invention in an effective amount to achieve an intended purpose. Determination of an effective amount and intended purpose is within the skill of the art. Preferred dosages are dependent for example, on the severity of the disease and the individual patient's response to the treatment.

As used herein, the term "pharmaceutically acceptable salts" is intended to mean salts of the compounds of the present invention with pharmaceutically acceptable acids, e.g., inorganic acids such as sulfuric, hydrochloric, phosphoric, etc. or organic acids such as acetic.

Pharmaceutically acceptable compositions of the present invention may also include suitable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which may be used pharmaceutically. Such preparations can be administered orally (e.g., tablets, dragees and capsules), rectally (e.g. suppositories), as well as administration by injection.

The pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, e.g., using the conventional mixing, granulating, dragee-making, dissolving or lyophilizing processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding a resulting mixture and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

Suitable excipients are, in particular, fillers such as sugars, e.g., lactose or sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, e.g., tricalcium diphosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, e.g., maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose and/or polyvinylpyrrolidone. If desired, disintegrating agents may be added such as the above-mentioned starches and also carboxymethyl starch, cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are, above all, flow-regulating agents and lubricants, e.g., silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate, and/or polyethylene glycol. Dragee cores are provided with suitable coatings which, if desired, are resistant to gastric juices. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvent or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations, such as acetylcellulose phthalate or hydroxypropylmethyl cellulose phthalate, are used. Dyestuffs or pigments may be added to the tablets or dragee coatings, e.g., for identification or in order to characterize different combinations of active compound doses.

Other pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules may contain the active compounds in the form of granules which may be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be used.

Possible pharmaceutical preparations which can be used rectally include, e.g., suppositories, which consist of a combination of the active compounds with a suppository base. Suitable suppository bases are, e.g., natural or synthetic triglycerides, paraffin hydrocarbons, polyethylene glycols or higher alkanols. It is also possible to use gelatin rectal capsules which consist of a combination of the active compounds with a base. Possible base materials include, e.g., liquid triglycerides, polyethylene glycols or paraffin hydrocarbons.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, e.g., water-soluble salts. In addition, suspension of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, such as sesame oil, or synthetic fatty acid esters, e.g., ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension such as sodium carboxymethylcellulose, sorbitol and/or dextran. Optionally, the suspension may also contain stabilizers.

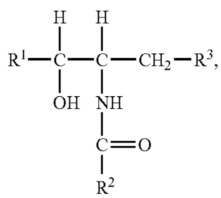
Alternatively, the active compounds of the present invention may be administered in the form of liposomes, pharmaceutical compositions wherein the active compound is contained either dispersed or variously present in corpuscles consisting of aqueous concentrate layers adherent to hydrophobic lipidic layer. The active compound may be present both in the aqueous layer and in the lipidic layer or in the non-homogeneous system generally known as a lipophilic suspension.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention.

All publications cited herein are expressly incorporated by reference.

What is claimed is:

1. A pharmaceutical composition comprising a compound represented by the following structural formula:



or a stereoisomer, a pharmaceutically acceptable salt or a mixture thereof; and

a pharmaceutically acceptable carrier or an excipient, wherein:

R^1 is a phenyl, a substituted phenyl group, a branched aliphatic group, or a 7-15 carbons long alkyl chain or a 7-15 carbons long alkenyl chain with a double bond next to the kernel;

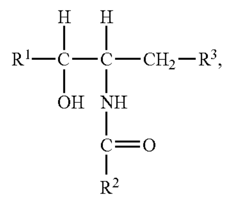
R^2 is an alkyl group 6, 7, or 8 carbons long; and

R^3 is a pyrrolidine, azetidine or piperidine, in which the nitrogen atom is attached to the kernel.

2. The pharmaceutical composition of claim **1**, wherein R^1 is a phenyl group substituted with a functional group

selected from the group consisting of a p-methoxy, hydroxyl, methylenedioxy, ethylenedioxy, trimethylenedioxy and cyclohexyl.

3. A pharmaceutical composition comprising a compound represented by the structural formula:



or a stereoisomer, a pharmaceutically acceptable salt or a mixture thereof; and

a pharmaceutically acceptable carrier or an excipient, wherein:

R^1 is a substituted or unsubstituted phenyl group, a branched aliphatic group, or a 7-15 carbons long alkyl chain or a 7-15 carbons long alkenyl chain with a double bond next to the kernel;

R^2 is an alkyl group 6, 7, or 8 carbons long; and

R^3 is pyrrolidine, in which the nitrogen atom is attached to the kernel.

4. The pharmaceutical composition of claim **3**, wherein R^1 is a phenyl group substituted with a functional group selected from the group consisting of a p-methoxy, hydroxyl, methylenedioxy, ethylenedioxy, trimethylenedioxy and cyclohexyl.

* * * * *

EXHIBIT D

US007615573B2

(12) **United States Patent**
Siegel et al.

(10) **Patent No.: US 7,615,573 B2**
(45) **Date of Patent: *Nov. 10, 2009**

(54) **SYNTHESIS OF UDP-GLUCOSE: N-ACYLSPHINGOSINE GLUCOSYLTRANSFERASE INHIBITORS**

(75) Inventors: **Craig Siegel**, Woburn, MA (US); **James A. Shayman**, Ann Arbor, MI (US); **Carol A. Nelson**, Westford, MA (US); **David J. Harris**, Lexington, MA (US); **Diane P. Copeland**, North Billerica, MA (US)

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 296 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **11/702,425**

(22) Filed: **Feb. 5, 2007**

(65) **Prior Publication Data**

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Related U.S. Application Data

(60) Division of application No. 11/091,836, filed on Mar. 28, 2005, now Pat. No. 7,196,205, which is a continuation of application No. 10/916,824, filed on Aug. 12, 2004, now Pat. No. 7,265,228, which is a division of application No. 10/197,227, filed on Jul. 16, 2002, now Pat. No. 6,855,830.

(60) Provisional application No. 60/305,814, filed on Jul. 16, 2001.

(51) **Int. Cl.**
A61K 31/40 (2006.01)
A61K 31/445 (2006.01)
A61K 31/5375 (2006.01)
C07D 405/06 (2006.01)

(52) **U.S. Cl.** **514/422**; 548/526

(58) **Field of Classification Search** 548/526; 514/422

See application file for complete search history.

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(57) **ABSTRACT**

Disclosed is a novel enantiomeric synthesis ceramide-like inhibitors of UDP-glucose: N-acylsphingosine glucosyltransferase. Also disclosed are novel intermediates formed during the synthesis.

30 Claims, 4 Drawing Sheets

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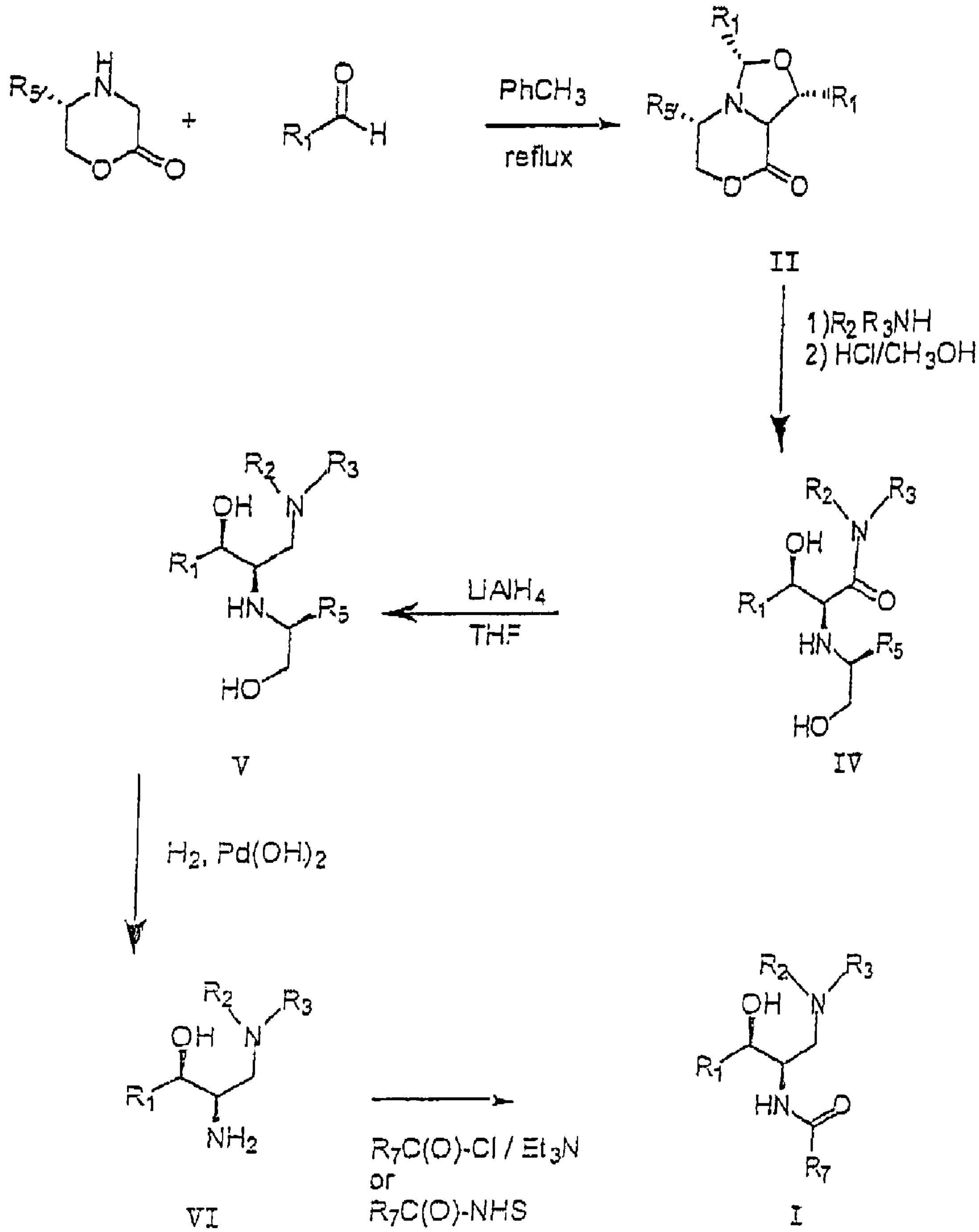
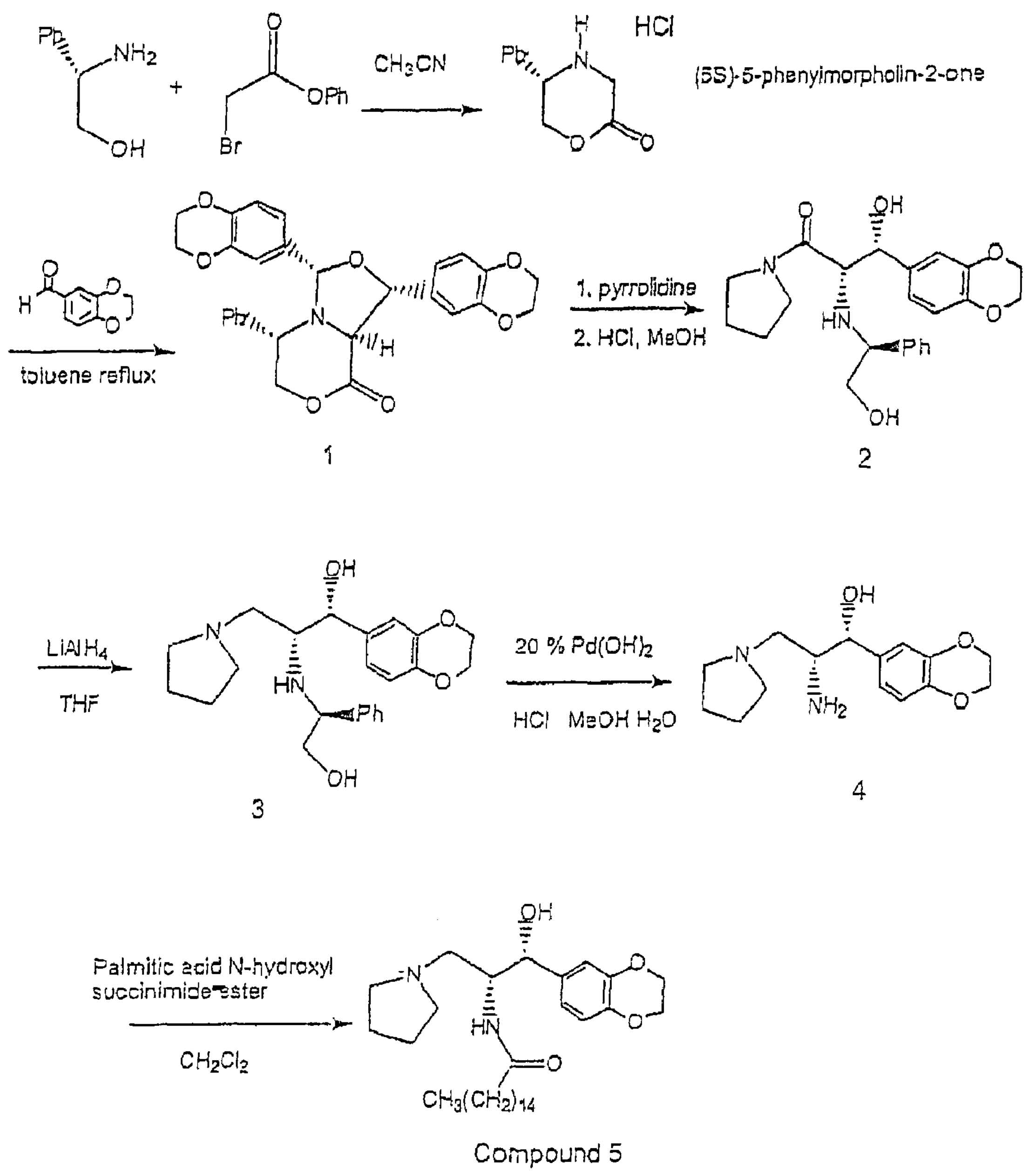


PhCH3
reflux
II
1) R2R3NH
2) HCl/CH3OH
IV
LiAlH4
THF
V
H2, Pd(OH)2
VI
R7C(O)-Cl / Et3N
or
R7C(O)-NHS
I

Figure 1



Ph
NH_2
OH
+
O
OPh
Br
CH_3CN
H
N
HCl
O
O
(5S)-5-phenylmorpholin-2-one
toluene reflux
1
1. pyrrolidine
2. HCl, MeOH
2
$LiAlH_4$
THF
3
20 % $Pd(OH)_2$
HCl MeOH H_2O
NH_2
4
Palmitic acid N-hydroxyl succinimide ester
CH_2Cl_2
$CH_3(CH_2)_{14}$
Compound 5

Figure 2

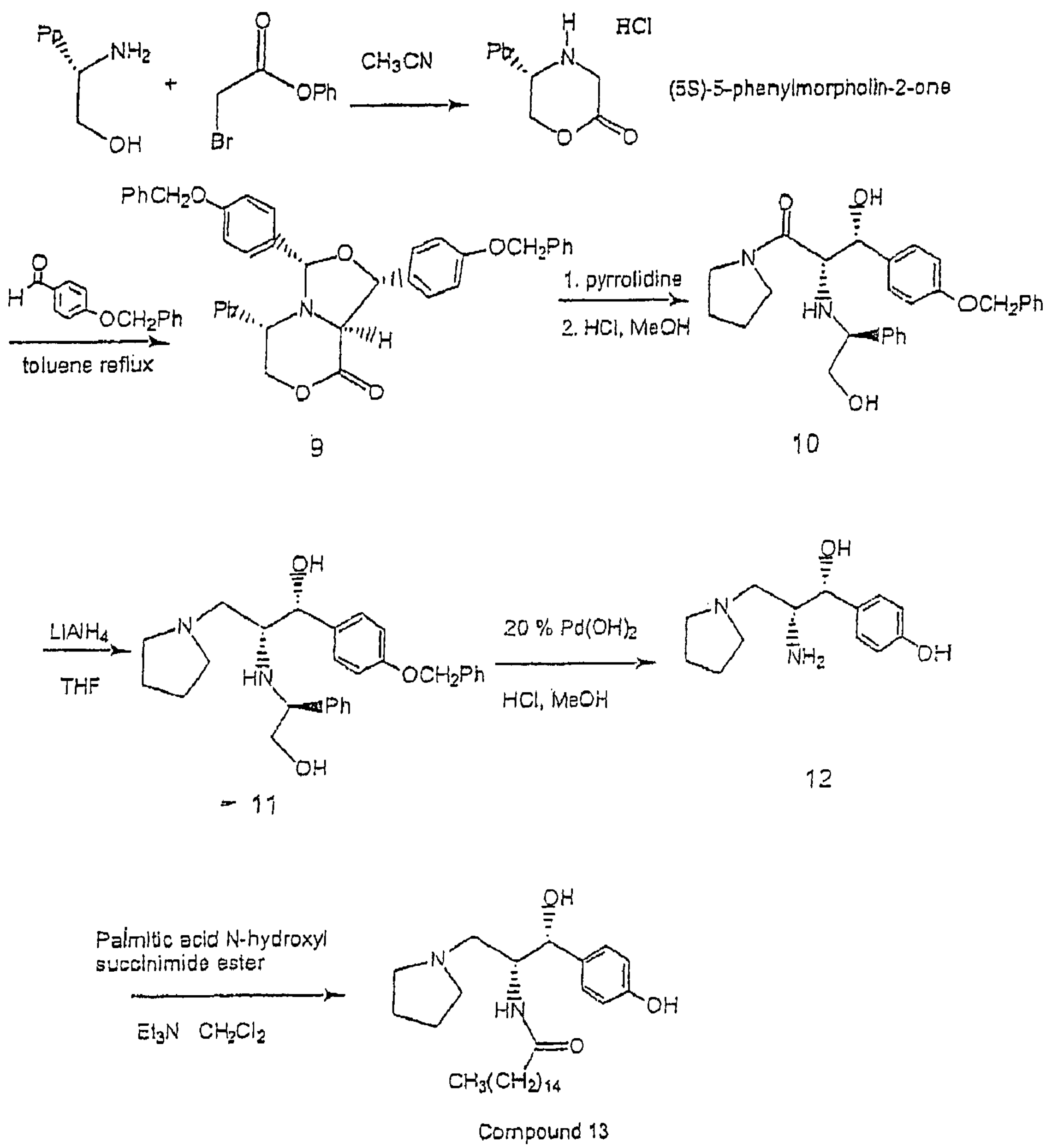
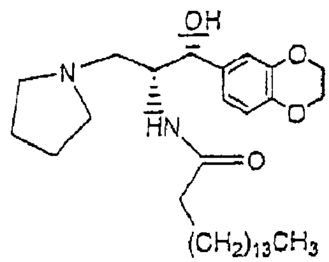
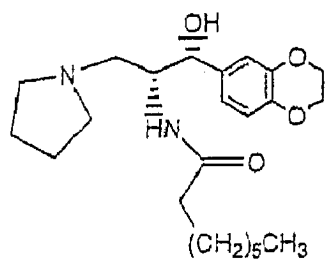

CH3CN
HCl
(5S)-5-phenylmorpholin-2-one
toluene reflux
1. pyrrolidine
2. HCl, MeOH
9
10
LiAlH4
THF
20 % Pd(OH)2
HCl, MeOH
11
12
Palmitic acid N-hydroxyl succinimide ester
Et3N CH2Cl2
Compound 13

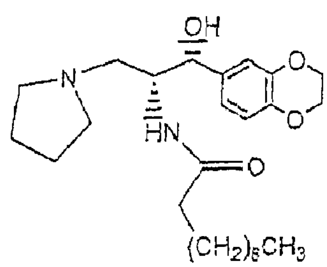
Figure 3



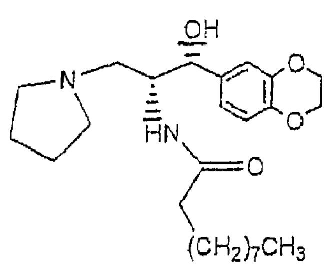
Compound 5



Compound 6



Compound 7



Compound 8

Figure 4

SYNTHESIS OF UDP-GLUCOSE: N-ACYLSPHINGOSINE GLUCOSYLTRANSFERASE INHIBITORS

RELATED APPLICATIONS

This application is a divisional of U.S. patent application Ser. No. 11/091,836, filed on Mar. 28, 2005, now U.S. Pat. No. 7,196,205, which is a continuation of U.S. patent application Ser. No. 10/916,824, filed Aug. 12, 2004, now U.S. Pat. No. 7,265,228, which is a divisional of U.S. patent application Ser. No. 10/197,227, filed Jul. 16, 2002, now U.S. Pat. No. 6,855,830, which claims the benefit of U.S. Provisional Application No. 60/305,814, filed Jul. 16, 2001. The entire teachings of these applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

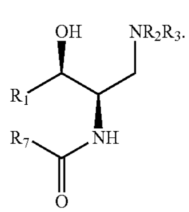
Glycosphingolipids (GSLs) are a class of naturally occurring compounds which have a multitude of biological functions, including the ability to promote cell growth, cell differentiation, adhesion between cells or between cells and matrix proteins, binding of microorganisms and viruses to cells, and metastasis of tumor cells. GSLs are derived from glucosylceramide (GlcCer), which is produced from ceramide and UDP-glucose by the enzyme UDP-glucose: N-acylsphingosine glucosyltransferase (GlcCer synthase). The structure of ceramide is shown below: crystallization after derivitization with optically active reagents, e.g., dibenzoyltartaric acid isomers (see, for example, Inokuchi and Radin, *Journal of Lipid Research* 28: 565 (1987)). Neither of the processes are amenable to large scale preparations. Enantioselective synthesis of amino ceramide-like compounds using diastereoselective reductions have been reported (Mitchell, et al., *J Org. Chem.* 63:8837 (1998) and Nishida, et al., *SYNLETT* 1998. 389 (1998)), but require over ten steps, some of which utilized expensive reagents such as diisobutylaluminum hydride (DIABAL) and Garner Aldehyde (tert-butyl (R)-(+)-4 formyl-2,2-dimethyl-3-oxazolidine carboxylate). Thus, there is a critical need for enantioselective syntheses of amino ceramide-like compounds which are more economical and efficient, and involve fewer steps than known syntheses.

SUMMARY OF THE INVENTION

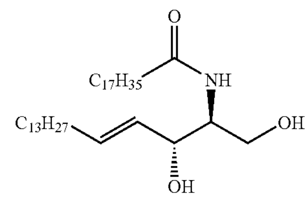
Provided herein is an efficient, highly enantioselective synthesis of amino ceramide-like compounds. This synthesis of amino ceramide-like compounds involves only five steps from known compounds. For example, the ceramide-like compound designated as "Compound 5" in FIG. **2** was produced in an enantiomeric excess of at least 99.6% and an overall yield of 9% (see Examples 1 and 2). Novel intermediates prepared during the course of the synthesis are also disclosed.

The present invention is directed is a method of preparing a ceramide-like compound represented by Structural Formula (I):

(I)



R_1 is a substituted or unsubstituted aromatic group; preferably, R_1 is a substituted or unsubstituted phenyl group, more preferably phenyl substituted in the



Ceramide

The accumulation of GSLs has been linked to a number of diseases, including Tay-Sachs, Gaucher, and Fabry diseases (see, for example, U.S. Pat. No. 6,051,598). GSLs have also been linked to certain cancers. For example, it has been found that certain GSLs occur only in tumors or at abnormally high concentrations in tumors; exert marked stimulatory or inhibitory actions on tumor growth when added to tumor cells in culture media; and inhibit the body's normal immunodefense system when shed by tumors into the surrounding extracellular fluid. The composition of a tumor's GSLs changes as the tumors become increasingly malignant and antibodies to certain GSLs inhibit the growth of tumors.

Compounds which inhibit GlcCer synthase can lower GSL concentrations and have been reported to be useful for treating a subject with one of the aforementioned diseases. A number of potent inhibitors of GlcCer, referred to herein as "amino ceramide-like compounds", are disclosed in U.S. Pat. Nos. 6,051,598, 5,952,370, 5,945,442, 5,916,911 and 6,030,995. The term "ceramide-like compounds" refers to analogs of ceramide in which: 1) the primary alcohol is replaced with a substituted amino group; and 2) the alkenyl group is replaced with an aryl group, preferably phenyl or substituted phenyl. The corresponding N-deacylated compounds are referred to as "sphingosine-like compounds."

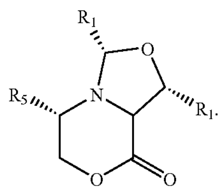
Unfortunately, known methods of preparing amino ceramide-like compounds are poorly suited for manufacturing on an industrial scale. Because of the two chiral centers, most known syntheses generate four diastereoisomers, resulting in the need to separate diastereomers by chromatography and to isolate the desired enantiomer by meta/para positions with —OCH_2O—, —OCH_2CH_2O— or in the para position with halo, lower alkyl thiol, —OH, —O(phenyl), —OCH_2(phenyl), lower alkyl, amino, lower alkyl amino, lower dialkyl amino, or —O(lower alkyl);

R_2 and R_3 are independently —H, a substituted or unsubstituted aliphatic group or, taken together with the nitrogen atom to which they are bonded, are a substituted or unsubstituted non-aromatic heterocyclic ring.

R_7 is a substituted or unsubstituted aliphatic group, preferably a C1-C30 straight chain unsubstituted aliphatic group or a C1-C30 straight chained aliphatic group substituted with one or more C1-C2 alkyl groups, more preferably an unsubstituted C1-C30 straight chain alkyl or alkenyl group and even more preferably an unsubstituted C7-C10 or C10-C16 straight chain alkyl or alkenyl group.

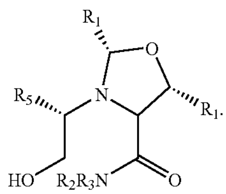
The method of preparing a ceramide-like compound represented by Structural Formula (I) comprises a first step whereby an amine compound HNR_2R_3 is reacted with a cyclic starting material represented by Structural Formula (II):

(II)



The reaction between the amine compound HNR_2R_3 and the cyclic starting material represented by Structural Formula (II) forms an amide intermediate represented by Structural Formula (III):

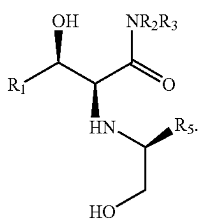
(III)



In Structural Formulas (II) and (III), R_1-R_3 are as described for Structural Formula (I); and R_5 is a substituted or unsubstituted aromatic group, preferably a substituted or unsubstituted phenyl group.

The method of preparing a ceramide-like compound represented by Structural Formula (I) comprises a second step whereby the amino acetal group in the intermediate represented by Structural Formula (III) is hydrolyzed to form the acyclic compound represented by Structural Formula (IV).

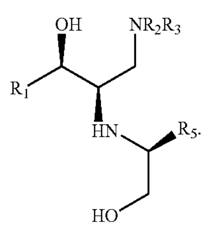
(IV)



R_1, R_2, R_3 and R_5 in Structural Formulas (IV) are as defined in Structural Formulas (I)-(III).

The method of preparing a ceramide-like compound represented by Structural Formula (I) comprises a third step whereby the acyclic precursor compound represented by Structural Formula (IV) is reacted with an amide reducing agent to form a compound represented by Structural Formula (V):

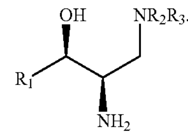
(V)



R_1, R_2, R_3 and R_5 in Structural Formula (V) are as defined in Structural Formulas (I)-(IV).

The method of preparing a ceramide-like compound represented by Structural Formula (I) comprises a fourth step whereby the —NHCH(—CH_2OH)R_5 group of the amine compound represented by Structural Formula (V) is debenzylated to form a sphingosine-like compound represented by Structural Formula (VI):

(VI)



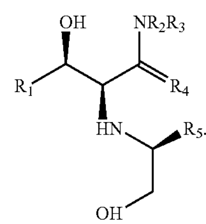
Preferably, the debenzylation is achieved by hydrogenation. R_1, R_2 and R_3 are as described for Structural Formulas (I)-(V).

The method of preparing a ceramide-like compound represented by Structural Formula (I) comprises a fifth step whereby the sphingosine-like compound represented by Structural Formula (VI) is acylated to form the ceramide-like compound represented by Structural Formula (I).

Other embodiments of the present invention include each of the individual reactions described above, taken separately and in combination with the other reactions.

Other embodiments of the present invention are intermediates in the preparation of the ceramide-like compound represented by Structural Formula (I) by the methods disclosed herein. In one example, the present invention is directed to an intermediate represented by Structural Formula (VII):

(VII)

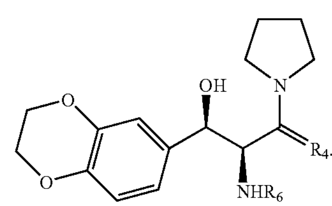


R_1-R_3 and R_5 are as described above for Structural Formulas (I)-(VI); and

R_4 is H_2 or O.

In another embodiment, the present invention is directed to an intermediate represented by Structural Formula (VIII):

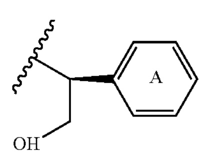
(VIII)



R_4 is H_2 or O; and

R_6 is represented by Structural Formula (IX):

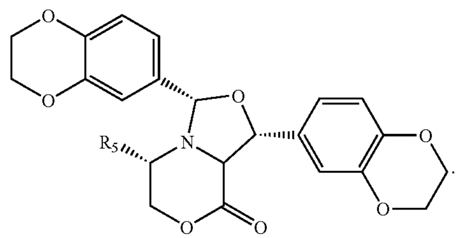
(IX)



Phenyl ring A in Structural Formula (IX) is substituted or unsubstituted. Preferably, however, phenyl ring A is unsubstituted. Alternatively, R_4 in Structural Formula (VIII) is H_2 and R_6 is —H.

In another embodiment, the present invention is directed to an intermediate represented by Structural Formula (X):

(X)



R_5 in Structural Formula (X) is as defined for Structural Formula (I).

The methods of the present invention can be utilized to prepare ceramide-like compounds that inhibit the enzyme GlcCer synthase in five steps from known starting materials. The synthesis is highly efficient, resulting in an overall yield that is generally greater than 8% and in an enantiomeric excess that is typically greater than 99%. The synthesis utilizes inexpensive reagents and therefore provides an economical route to potent inhibitors of GlcCer synthase.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic showing the synthesis of ceramide-like compounds represented by Structural Formula (I) using the methods and intermediates disclosed herein.

FIG. **2** is a schematic showing the synthesis of ceramide-like Compound (5) using the methods disclosed herein.

FIG. **3** is a schematic showing the synthesis of ceramide-like compound (13) using the methods disclosed herein.

FIG. **4** shows the structures of Compounds (5)-(8).

DETAILED DESCRIPTION OF THE INVENTION

Described herein is a five step synthesis of amino ceramide-like compounds from known starting materials. The synthesis begins with the preparation of the cyclic starting material represented by Structural Formula (II). The cyclic starting material is reacted with a suitable amine, thereby opening the lactone ring and forming the amide intermediate represented by Structural Formula (III). The amino acetal in the amide intermediate is hydrolyzed to form the acyclic compound represented by Structural Formula (IV). The amide of this acyclic compound is reduced with an amide reducing agent to form an amine compound represented by Structural Formula (V), which is in turn debenzylated to form the sphingosine-like compound represented by Structural Formula (VI). The primary amine of the sphingosine-like compound represented by Structural Formula (VI) can then be acylated to form an amino ceramide-like compound. This synthesis is shown schematically in FIG. **1**. A detailed description of each reaction in the synthesis is provided below.

The cyclic starting material represented by Structural Formula (II) is prepared according to methods described in Alker, et al., *Tetrahedron* 54:6089 (1998) and Harwood and Robertson, *Chem. Commun.* 1998:2641 (1998). Specifically, (5S)-5-phenylmorpholin-2-one is reacted with at least two equivalents and preferably from about 2.5 to about 5.0 equivalents of aryl aldehyde R_1CHO under dehydrating conditions. R_1 is as defined in Structural Formula (I). "Dehydrating conditions" refer to conditions under which water is removed from the reaction mixture. Removal of water can be achieved, for example, by carrying out the reaction in presence of a reagent (a "dehydrating reagent") that reacts with water (e.g., molecular sieves) but is substantially inert towards the other reagents present in the reaction mixture, or removal of water can also be achieved by azeotroping with a solvent such as toluene. Sufficient dehydrating reagent is used to remove the two equivalents of water (relative to cyclic starting material) released during the reaction. The concentration of reagents if typically between about 0.01 M and about 5.0 M, more typically between about 0.1 M and about 1.0 M; suitable reaction temperatures range between about 50° C. and about 150° C., preferably between about 100° C. and about 120° C.

The cyclic starting material is converted to the amide intermediate represented by Structural Formula (II) by reacting the cyclic starting material with the amine NHR_2R_3 under conditions suitable for amidating an ester with an amine. Such conditions are well known in the art and are described, for example, in March, "Advanced Organic Chemistry—Reactions, Mechanisms and Structure", Third Edition, John Wiley & Sons, 1985, pages 375-76, and references cited therein. Although an excess of either reagent can be used, cyclic starting material is more commonly the limiting reagent. Generally up to about fifteen equivalents of amine relative to cyclic starting material are used, typically up to about eight equivalents. The reaction can be done neat, however, it is more usually carried out in a aprotic, non-nucleophilic solvent at amine concentrations as dilute as 0.01 M. Amine concentrations are more typically, however, between about 0.4 M and about 4.0 M. Suitable solvents include halogenated solvents such as chloroform, dichloromethane and 1,2-dichloroethane, acetonitrile, dimethylformamide (DMF), ethereal solvents such as diethyl ether, tetrahydrofuran (THF) and 1,4-dioxane and aromatic solvents such as benzene and toluene. Suitable reaction temperatures generally range from about 0° C. to about 100° C., typically between about 25° C. to about 35° C.

Conditions for hydrolyzing aminoacetals are known in the art and are described, for example, in March, "Advanced Organic Chemistry—Reactions, Mechanisms and Structure", Third Edition, John Wiley & Sons, 1985, pages 329-32, and references cited therein. For example, the aminoacetal group in the amide intermediate represented by Structural Formula (III) can be hydrolyzed with dilute aqueous mineral acid. Suitable acids include hydrochloric acid, sulfuric acid or phosphoric acid, although hydrochloric is the most common choice. Organic acids such as acetic acid and sulfonic acids (e.g., methansulfonic acid, toluenesulfonic acid, trifluormethylsulfonic acid and the like) can also be used. At least one equivalent of acid relative to the intermediate is typically used, but an excess of acid is preferred to ensure complete hydrolysis, for example, excesses of at least ten fold, preferably an excess of about two to about three fold and more preferably between about 10-50%. The concentration of acid in the reaction mixture is generally between about 0.05 M to about 1.0 M, typically between about 0.1 M and about 0.5 M. An organic co-solvent miscible with water is often used to solubilize the intermediate. Examples include alcohols such as methanol or ethanol and DMF. Common solvent ratios of organic solvent to water range between about 1:1 to about 8:1. Suitable reaction temperatures range from ambient temperature to about 100° C., preferably between about 60° C. to about 80° C. Alternatively, the amino acetal can be hydrolyzed with Lewis acids such as trimethylsilyl iodide, wet silica gel or $LiBF_4$ in wet acetonitrile, as described in March, supra.

An "amide reducing agent" is a reagent which can reduce an amide to an amine. Such reagents are known in the art and are disclosed in, for example, in March, "Advanced Organic Chemistry—Reactions, Mechanisms and Structure", Third Edition, John Wiley & Sons, 1985, pages 1099-1100, Brown and Krishnamurthy, *Aldrichimica Acta* 12:3 (1979) and references cited therein. Examples include lithium aluminum hydride, lithium triethyl borohydride, borane reagents (e.g., borane•tetetrahydrofuran, borane•methyl sulfide, disiamylborane, and the like), aluminum hydride, lithium trimethoxy aluminum hydride and triethyloxonium fluoroborate/sodium borohydride. In the method of the present invention, lithium aluminum hydride is the most commonly used amide reducing agent. Although as little as 0.5 equivalents of lithium aluminum hydride relative to amide starting material can be used, it is more common to use an excess, often up to about five equivalents. Preferably, between about 1.5 and about 2.5 equivalents of lithium aluminum hydride are used relative to amine starting material. Ethereal solvents are typically used for the reduction; examples include diethyl ether, THF, glyme, diglyme and 1,4-dioxane. Suitable concentrations of reducing agent are generally between about 0.1 M and about 5.0 M, more typically between about 0.8 M and about 1.5 M. The reduction is most commonly carried out at ambient temperature, but temperatures between about 0° C. and about 80° C. or 100° C. can also be used.

To form the sphingosine-like compound represented by Structural Formula (VI), the amine compound represented by Structural Formula (V) is debenzylated. The term "debenzylating" is used herein to refer to cleaving the carbon-nitrogen bond of a group —NH—CH_2Z, wherein Z is an aryl group, preferably phenyl. Optionally, the methylene group can be replaced with a methine group. With respect to the sphingosine-like compound represented by Structural Formula (VI), "debenzylation" refers to converting the —NH CH(—CH_2OH)R_5 group to —NH_2, Debenzylation conditions are well known in the art and are disclosed, for example, in Greene and Wuts, "*Protective Groups in Organic Synthesis*", John Wiley & Sons (1991), pages 384-86 and references cited therein.

Preferably, debenzylation is achieved by hydrogenation under a hydrogen atmosphere and in the presence of a hydrogenation catalyst. Suitable hydrogen pressures are generally between about atmospheric pressure and about 1000 pounds per square inch. Other sources of hydrogen (e.g., formic acid, ammonium formate, cyclohexene and the like) can also be used. Suitable hydrogenation catalysts include 20% palladium hydroxide on carbon (Perlman's catalyst), palladium chloride, palladium, platinum oxide and palladium on carbon. Typically, between about 10% and about 100% weight/weigh (w/w) relative to amine compound is used. In most instances, an organic acid such as formic acid, acetic acid or trifluoroacetic acid or an inorganic acid such as hydrochloric acid or sulfuric acid is present, for example, between about one to about five equivalents relative to amine compound, preferably between about 1.6 to about 2.4 equivalents. The reaction is most commonly carried out in an alcoholic solvent such as methanol or ethanol with water as co-solvent (e.g., between 0% and about 50% volume/volume (v/v), preferably between about 5% and about 15% v/v). Reaction temperatures between about 0° C. and about 50° C. are suitable, preferably between about 25° C. and about 40° C.

Many debenzylation conditions other than hydrogenation are known in the art and are included in the present invention. Examples include sodium metal and NH_3 (see, for example, du Vigneaud and Behrens, *J Biol. Chem.* 117:27 (1937)), CCl_3CH_2OCOCl, CH_3CN (see, for example, Rawal, et al., *J Org. Chem.,* 52:19 (1987)), $Me_3SiCH_2CH_2OCOCl$, THF, −50° C., then 25° C. overnight (see, for example, Campbell, et al., *Tetrahedron Lett.,* 28:2331 (1987)), α-chloroethyl chloroformate and sodium hydroxide (see, for example Olofson, et al., *J Org. Chem.* 49:2081 (1984) and DeShong and Kell, *Tetrahedron Lett.,* 27:3979 (1986)), vinyl chloroformate (see, for example, Olofson et al., *Tetrahedron Lett.,* 1977:1567 (1977) and Cooley and Evain, *Synthesis,* 1989:1 (1989)), RuO_4, NH_3, H_2O (see, for example, Gao and Jones, *J Am. Chem. Soc.,* 109:1275 (1987)) and m-chloroperoxybenzoic acid followed by $FeCl_2$, −10° C. (see, for example, Monkovic, et al., *Synthesis,* 1985:770 (1985).

The sphingosine-like compound represented by Structural Formula (VI) is converted to a ceramide-like compound by acylating the free amine. Acylations of amine groups are well known in the art and can be carried out, for example, by reacting the amine with an acylating agent R_7C(O)—X. R_7 is as described above for Structural Formula (I) and X is a leaving group that is readily displaced by a primary amine. Conditions for this reaction are described in, for example, in March, "Advanced Organic Chemistry—Reactions, Mechanisms and Structure", Third Edition, John Wiley & Sons, 1985 and references cited therein. Examples of suitable acylating agents include acid halides, anhydrides or esters. Preferably, the amine is acylated with an acid chloride. Generally, equimolar amounts of the sphingosine-like compound and the acid chloride are used in the presence of a small excess, relative to the acid chloride, of a tertiary amine such as triethylamine, diisopropylethylamine, dimethylaminopyridine or pyridine is used. However, an excess of acid chloride (typically about 10-50%) can be used when the sphingosine-like compound is limiting, and vice versa. The concentrations of the reagents in the reaction mixture normally vary between about 0.005 M and about 5.0 M, and are preferably between about 0.05 M and about 0.5 M. The excess of amine base can be greater than about 100%, but is typically between about 5% and about 25%. Aprotic solvents such as halogenated solvents are preferred (e.g., chloroform, methylene chloride and 1,2-dichloromethane), however other aprotic solvents such as ethereal solvents and hydrocarbon solvents can be suitable substitutes. Ambient temperature is normally preferred for the reaction, but temperatures between about 0° C. and about 50° can also be used.

Alternatively, the acylating agent is an activated ester R_7C(O)—OX', wherein —OX' is readily displaced by a primary amine. Methods of acylating an amine with activated esters are known in the art and are described in, for example, March, "Advanced Organic Chemistry—Reactions, Mechanisms and Structure", Third Edition, John Wiley & Sons, 1985, pages 371-375, and references cited therein. Many activated esters are stable enough to be isolated. N-Hydroxy succinimidyl esters, some of which are commercially available from Aldrich Chemical Co., Milwaukee, Wis., are one example of activated esters of this type. Conditions suitable for forming an amide with an acid chloride acylating agent, described in the prior paragraph, can typically be used with a stable activated ester. In contrast with acid chlorides, which require activation with tertiary amines, activated esters are reactive enough so that they form amides directly in the presence of primary amines. Therefore, the tertiary amine can be omitted from the acylation reaction when activated esters are used.

Alternatively, an activated ester is formed in situ. Formation of an activated ester in situ requires a "coupling agent", which is a reagent that replaces the hydroxyl group of a carboxyl acid with a group which is susceptible to nucleophilic displacement. Examples of coupling agents include 1,1'-carbonyldiimidazole (CDI), isobutyl chloroformate,

dimethylaminopropylethyl-carbodiimide (EDC), dicyclohexyl carbodiimide (DCC). When amidating by in situ generation of an activated ester, an excess of either the carboxylic acid or amine can be used (typically a 50% excess, more typically about a 10-15% excess). However, it is more common when carrying out the present invention to use the amine compound as the limiting reagent. Generally, from about 1.0 mole to about 10 moles of coupling agent are used per mole of carboxylic acid, preferably from about 1.0 mole to about 1.5 moles of coupling agent per mole of carboxylic acid. The reaction is generally carried out in aprotic solvents, for example, halogenated solvents such as methylene chloride, dichloroethane and chloroform, ethereal solvents tetrahydrofuran, 1,4-dioxane and diethyl ether and dimethylformamide. Suitable reaction temperatures generally range from between about 0° to about 100° C., but the reaction is preferably carried out at ambient temperature.

Examples of specific conditions for carrying out the reactions described herein are provided in Examples 1 and 2.

By utilizing the enantiomer of the compound represented by Structural Formula (II) as the cyclic starting material, the enantiomer of the compounds represented by Structural Formulas (III)-(VI) and (I) can be prepared by utilizing the methods described herein. The enantiomer of the cyclic starting material represented by Structural Formula (III) can be prepared by reacting (5R)-5-phenylmorpholin-2-one with two equivalents of the aldehyde R_1CHO under dehydrating conditions, as described above. The enantiomer of compounds represented by Structural Formula (III), (VII), (VIII) and (X) and methods of preparing the enantiomers of the compounds represented by Structural Formulas (II)-(VI) and (I) using procedures disclosed herein are encompassed within the present invention.

The term "enantiomer" as it used herein, and structural formulas depicting an enantiomer are meant to include the "pure" enantiomer free from its optical isomer as well as mixtures of the enantiomer and its optical isomer in which the enantiomer is present in an enantiomeric excess, e.g., at least 10%, 25%, 50%, 75%, 90%, 95%, 98%, or 99% enantiomeric excess.

With regard to the variables R_1 -R_5 in Structural Formulas (I)-(IX), an "aliphatic group" is non-aromatic, consists solely of carbon and hydrogen and may optionally contain one or more units of unsaturation, e.g., double and/or triple bonds. An aliphatic group may be straight chained, branched or cyclic. When straight chained or branched, an aliphatic group typically contains between about 1 and about 30 carbon atoms, more typically between about 1 and about 24 carbon atoms. When cyclic, an aliphatic group typically contains between about 3 and about 10 carbon atoms, more typically between about 3 and about 7 carbon atoms. Aliphatic groups are preferably lower alkyl groups, which include C1-30 straight chained or branched saturated hydrocarbons, preferably C1-C24 straight chained or branched saturated hydrocarbons. Examples include methyl, ethyl, n-propyl, iso-propyl, n-butyl, sec-butyl and tert-butyl.

Aromatic groups include carbocyclic aromatic groups such as phenyl, 1-naphthyl, 2-naphthyl, 1-anthracyl and 2-anthacyl, and heterocyclic aromatic groups such as N-imidazolyl, 2-imidazole, 2-thienyl, 3-thienyl, 2-furanyl, 3-furanyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidy, 4-pyrimidyl, 2-pyranyl, 3-pyranyl, 3-pyrazolyl, 4-pyrazolyl, 5-pyrazolyl, 2-pyrazinyl, 2-thiazole, 4-thiazole, 5-thiazole, 2-oxazolyl, 4-oxazolyl and 5-oxazolyl.

Aromatic groups also include fused polycyclic aromatic ring systems in which a carbocyclic aromatic ring or heteroaryl ring is fused to one or more other heteroaryl rings. Examples include 2-benzothienyl, 3-benzothienyl, 2-benzofuranyl, 3-benzofuranyl, 2-indolyl, 3-indolyl, 2-quinolinyl, 3-quinolinyl, 2-benzothiazole, 2-benzooxazole, 2-benzimidazole, 2-quinolinyl, 3-quinolinyl, 1-isoquinolinyl, 3-quinolinyl, 1-isoindolyl and 3-isoindolyl.

Non-aromatic heterocyclic rings are non-aromatic carbocyclic rings which include one or more heteroatoms such as nitrogen, oxygen or sulfur in the ring. The ring can be five, six, seven or eight-membered. Examples include morpholinyl, thiomorpholinyl, pyrrolidinyl, piperazinyl, piperidinyl, azetidinyl, azacycloheptyl, or N-phenylpiperazinyl.

Suitable substituents on a lower alkyl, aliphatic, aromatic, non-aromatic, heterocyclic or benzyl group are those which do not substantially interfere with the reactions described herein. "Interfering with a reaction" refers to substantially decreasing the yield (e.g., a decrease of greater than 50%) or causing a substantial amount of by-product formation (e.g., where by-products represent at least 50% of the theoretical yield). Interfering substituents can be used, provided that they are first converted to a protected form. Suitable protecting groups are known in the art and are disclosed, for example, in Greene and Wuts, "Protective Groups in Organic Synthesis", John Wiley & Sons (1991).

Suitable substituents on an alkyl, aliphatic, aromatic, non-aromatic heterocyclic ring or benzyl group include, for example, halogen (—Br, —Cl, —I and —F), —OR, —CN, —NO_2, —NR_2, —COOR, —$CONR_2$, —SO_kR (k is 0, 1 or 2) and —NH—C(═NH)—NH_2. Each R is independently —H, an aliphatic group, a substituted aliphatic group, a benzyl group, a substituted benzyl group, an aromatic group or a substituted aromatic group, and preferably —H, a lower alkyl group, a benzylic group or a phenyl group. A substituted non-aromatic heterocyclic ring, benzylic group or aromatic group can also have an aliphatic or substituted aliphatic group as a substituent. A substituted alkyl or aliphatic group can also have a non-aromatic heterocyclic ring, benzyl, substituted benzyl, aromatic or substituted aromatic group as a substituent. A substituted alkyl, substituted aliphatic, substituted non-aromatic heterocyclic, substituted aromatic or substituted benzyl group can have more than one substituent.

When R_1 is a substituted phenyl group, examples of preferred substituents include —OCH_2O—, —OCH_2CH_2O—, halo, (lower alkyl)O—, lower alkyl thiol, lower dialkylamine, —OH, —O(phenyl), —OCH_2 (phenyl), lower alkyl, amine and lower alkyl amino.

When R_5 is a substituted phenyl group, examples of preferred substituents include halo, (lower alkyl)O—, —O(phenyl) and lower alkyl.

In the structural formulas depicted herein, the remainder of the molecule or compound to which a chemical group or moiety is connected is indicated by the following symbol:

For example, the corresponding symbol in Structural Formula (IX) indicates that the depicted group, which is represented by R_6 in Structural Formula (VIII), is connected via the benzylic carbon to the amine in Structural Formula (VIII) by a single covalent bond.

In preferred embodiments of the present invention the variables used herein are defined as follows: R_1 is a substituted or unsubstituted phenyl group; R_2 and R_3 are independently —H, an unsubstituted C1-C5 alkyl group or, taken together with the nitrogen atom to which they are bonded, are an unsubstituted C3-C10 non-aromatic heterocyclic ring; R_5 is a substituted or unsubstituted phenyl group, preferably phenyl; and R_7 is a C1-C30 straight chain unsubstituted aliphatic group or a C1-C30 straight chained aliphatic group substi-

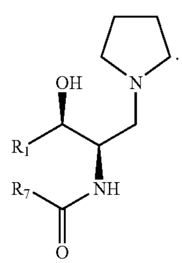
tuted with one or more C1-C2 alkyl group and more preferably an unsubstituted C1-C30 straight chain alkyl or alkenyl group.

In another preferred embodiment, —NR_2R_3, taken together, is pyrrolidinyl. More preferably, —NR_2R_3, taken together, is pyrrolidinyl and R_5 is phenyl in compounds comprising R_2, R_3 and R_5. Even more preferably, in compounds comprising R_1, R_2, R_3 and R_5, R_1 is a substituted or unsubstituted phenyl group (preferably phenyl substituted in the meta/para positions with —OCH_2O—, —OCH_2CH_2O—or in the para position with halo, lower alkyl thiol, —OH, —O(phenyl), —O—CH_2(phenyl), lower alkyl, amino, lower alkyl amine, lower dialkyl amino, or —O(lower alkyl), —NR_2R_3, taken together, is pyrrolindinyl and R_5 is phenyl.

In another preferred embodiment, —NR_2R_3, taken together, is piperidyl. More preferably, —NR_2R_3, taken together, is piperidyl and R_5 is phenyl in compounds comprising R_2, R_3 and R_5. Even more preferably, in compounds comprising R_1, R_2, R_3 and R_5, R_1 is a substituted or unsubstituted phenyl group (preferably phenyl substituted in the meta/para positions with —OCH_2O—, —OCH_2CH_2O— or in the para position with halo, lower alkyl thiol, —OH, —O(phenyl), —OCH_2-(phenyl), —OCH_2 (phenyl), lower alkyl, amino, lower alkyl amino, lower dialkyl amino, or —O(lower alkyl), —NR_2R_3, taken together, is piperidyl and R_5 is phenyl.

Examples of ceramide-like compounds which can be prepared by the methods of the present invention are represented by Structural Formula (XI):

(XI)



R_1 is phenyl substituted in the meta/para positions with —OCH_2O— or —OCH_2CH_2O— or in the para position with halo, CH_3O—, CH_3CH_2O—, $CH_3CH_2CH_2O$—, $CH_3(CH_3)CHO$—, CH_3—, CH_3CH_2—, $CH_3CH_2CH_2$—, $CH_3(CH_3)CH$—, —OH or —OCH_2(phenyl); and R_7 is $CH_3(CH_2)_n$— or $CH_3(CH_2)_{n-2}CH{=}CH$—, wherein n is an integer from 0 to about 30. Preferably, n is 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24. More preferably, R_1 is phenyl substituted meta/para with —OCH_2CH_2O—.

Compounds of this invention which possess a sufficiently acidic, a sufficiently basic, or both functional groups, and accordingly can react with any of a number of inorganic bases, and inorganic and organic acids, to form a salt. Thus, the present invention also includes salts of the intermediates represented by Structural Formulas (VII), (VIII) and (X). Physiologically acceptable salts are preferred. Acids commonly employed to form acid addition salts are inorganic acids such as hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, phosphoric acid, and the like, and organic acids such as p-toluenesulfonic acid, methanesulfonic acid, oxalic acid, p-bromophenyl-sulfonic acid, carbonic acid, succinic acid, citric acid, benzoic acid, acetic acid, and the like. Examples of such salts include the sulfate, pyrosulfate, bisulfate, sulfite, bisulfite, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, chloride, bromide, iodide, acetate, propionate, decanoate, caprylate, acrylate, formate, isobutyrate, caproate, heptanoate, propiolate, oxalate, malonate, succinate, suberate, sebacate, fumarate, maleate, butyne-1,4-dioate, hexyne-1,6-dioate, benzoate, chlorobenzoate, methylbenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, phthalate, sulfonate, xylenesulfonate, phenylacetate, phenylpropionate, phenylbutyrate, citrate, lactate, gamma-hydroxybutyrate, glycolate, tartrate, methanesulfonate, propanesulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, mandelate, and the like.

Base addition salts include those derived from inorganic bases, such as ammonium or alkali or alkaline earth metal hydroxides, carbonates, bicarbonates, and the like. Such bases useful in preparing the salts of this invention thus include sodium hydroxide, potassium hydroxide, ammonium hydroxide, potassium carbonate, and the like.

The entire teachings of the publications cited in this application are incorporated herein by reference.

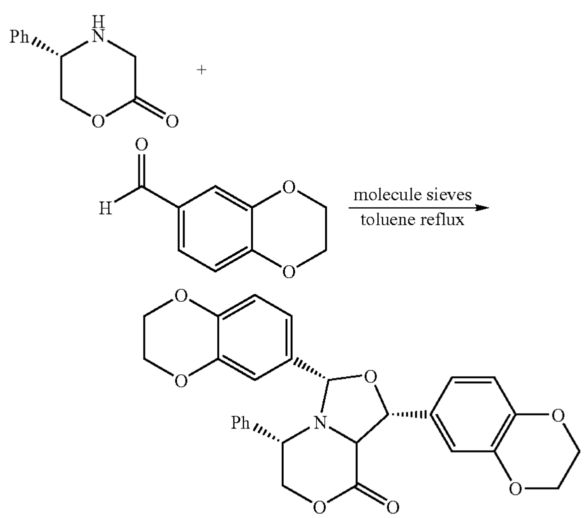
EXEMPLIFICATION

Example 1

Small Scale Preparation of Ceramid-Like Compounds

Intermediate 1

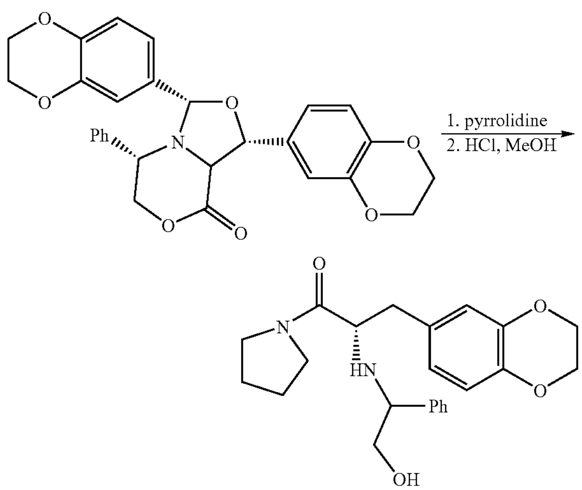
(1R,3S,5S,8aS)-1,3-Bis-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-5-phenyl-tetrahydro-oxazolo[4,3-c][1,4]oxazin-8-one



To a stirred solution of (5S)-5-phenylmorpholim-2-one (2.00 g, 11.3 mmol) (prepared as in: Dellaria, J. F.: Santarsiero, B. D. *J Org. Chem.,* 1989, 54, 3916) and 1,4-benzodioxan-6-carboxaldehyde (5.56 g, 33.9 mmol) in toluene (125 mL) was added 4 Å molecular sieves (approximately 20 mL). The mixture was heated at reflux for 72 hours, filtered free of sieves and concentrated. The resulting amber gum was flash chromatographed over silica (diethyl ether/hexane) to furnish a pale yellow solid. This material was further purified by trituration with diethyl ether to afford 1.89 g (34%) product as a fluffy white solid: ^1H NMR ($CDCl_3$) δ 7.31-7.17 (m, 5H), 6.95-6.79 (m, 5H), 5.32-5.27 (m, 2H), 4.43-4.28 (m, 2H), 4.24 (s, 4H), 4.18 (m, 4H), 4.16-4.08 (m, 2H) ppm.

Intermediate 2

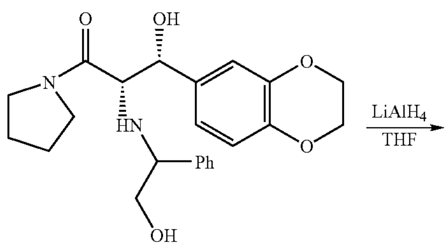
(2S,3R,1"S)-3-(2',3'-Dihydro-benzo[1,4]dioxin-6'-yl)-3-hydroxy-2-(2"-hydroxy-1"-phenyl-ethylamino)-1-pyrrolidin-1-yl-propan-1-one



To a stirred solution of Intermediate 1 (1.80 g, 3.69 mmol) in chloroform (20 mL) was added pyrrolidine (2.0 mL, 24 mmol). The solution was stirred overnight and then concentrated. The resulting colorless tacky foam was taken up in methanol (16 mL) and 1 N hydrochloric acid (4 mL). The mixture was refluxed for 1 hour, treated with additional 1 N hydrochloric acid (2 mL) and refluxed for another 2 hours. The reaction solution was concentrated and the resulting residue partitioned between ethyl acetate and aqueous sodium bicarbonate solution. The organic layer was dried (sodium sulfate) and concentrated. The resulting pale yellow gum was purified by flash chromatography over silica gel (methylene chloride/2 N methanolic ammonia) to afford 1.40 g (92%) of Intermediate 2 as a colorless foamy solid: ^{1}H NMR ($CDCl_3$) δ 7.31-7.13 (m, 5H), 6.93-6.70 (m, 3H), 4.47 (d, J=8.5, 1H), 4.18 (s, 4H), 3.82 (t, J=5.9, 1H), 3.74 (d, J=6.0, 2H), 3.06 (d, J=8.5, 1H), 3.06-2.97 (m, 1H), 2.92-2.83 (m, 1H), 1.97-1.87 (m, 1H), 1.45-1.15 (m, 4H) ppm.

Intermediate 3

(1R,2R,1"S)-1-(2',3'-Dihydro-benzo[1,4]dioxin-6'-yl)-2-(2"-hydroxy-1"-phenyl-ethylamino)-3-pyrrolidin-1-yl-propan-1-ol



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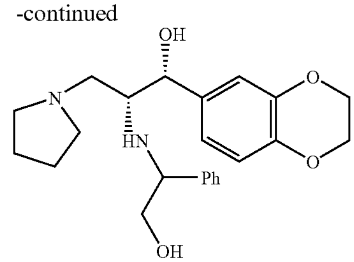
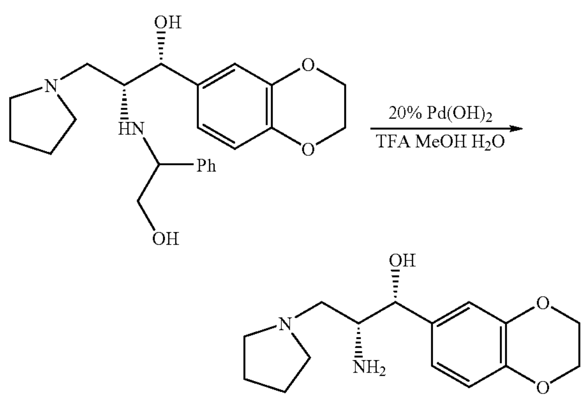


To a stirred solution of Intermediate 2 (1.38 g, 3.35 mmol) in tetrahydrofuran (30 mL) was added lithium aluminum hydride (0.26 g, 6.9 mmol). The foamy suspension was stirred overnight and then quenched with the addition (dropwise until frothing ceases) of 1 N aqueous sodium hydroxide (13 mL). The mixture was diluted with water and extracted with ethyl acetate. The organic layer was dried (sodium sulfate) and concentrated to afford a colorless gum. Flash chromatography over silica gel (methylene chloride/2 N methanolic ammonia) afforded 0.94 g (70%) of product as a colorless tacky foam: ^{1}H NMR ($CDCl_3$) δ 7.36-7.17 (m, 5H), 6.88-6.74 (m, 3H), 4.42 (d, J=5.4, 1H), 4.26 (s, 4H), 3.79-3.69 (m, 1H), 3.64-3.56 (m, 1H), 3.55-3.45 (m, 1H), 3.00-2.90 (m, 1H), 2.67-2.57 (m, 1H), 2.43-2.32 (m, 4H), 2.25-2.15 (m, 1H), 1.75-1.65 (m, 4H) ppm.

Intermediate 4

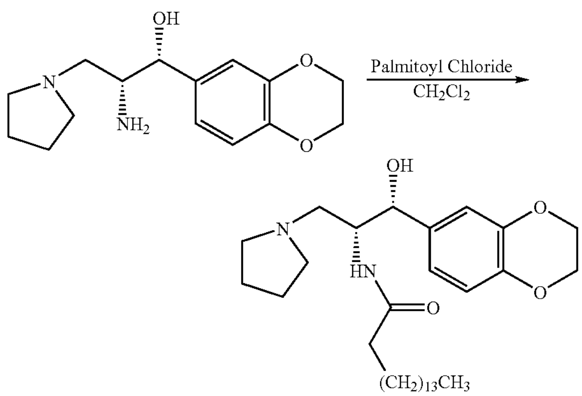
(1R,2R)-2-Amino-1-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-3-pyrrolidin-1-yl-propan-1-ol



In a high pressure reaction bomb equipped with a mechanical stirrer was loaded a solution of Intermediate 3 (0.91 g, 2.28 mmol) in 10:1 methanol/water (22 mL), trifluoroacetic acid (0.18 mL, 2.3 mmol) and 20% palladium hydroxide on carbon (Perlman's catalyst; 0.91 g). The reactor was evacuated and backfilled with argon three times and then evacuated and refilled with hydrogen (100 psi). The reaction was stirred for 2 days and then evacuated and flushed with nitrogen. The reaction solution was filtered through Celite and concentrated. The resulting gray-green gum was flash chromatographed over silica gel (methylene chloride/2 N methanolic ammonia) to afford 0.165 g (26%) of product as a near colorless gum: ^{1}H NMR ($CDCl_3$) δ 6.89-6.76 (m, 3H), 4.54 (d, J=3.7, 1H), 4.25 (s, 4H), 3.43 (s, 1H), 3.14-3.07 (m, 1H), 2.68-2.41 (m, 6H), 1.82-1.71 (m, 4H) ppm.

Compound 5

(1R,2R)-Hexadecanoic acid [2-(2',3'-dihydro-benzo [1,4]dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-ylm-ethyl-ethyl]-amide

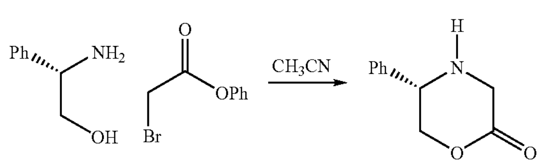


To a stirred solution of Intermediate 4 (0.165 g, 0.593 mmol) in methylene chloride (8 mL) was added palmitoyl chloride (0.18 g, 0.59 mmol) followed by N,N-diisopropylethylamine (0.11 mL, 0.65 mmol). The solution was stirred for 2 hours and then concentrated. The residue was partitioned between ethyl acetate and aqueous sodium bicarbonate solution. The organic layer was dried (sodium sulfate) and concentrated. The resulting off-white solid was flash chromatographed over silica gel (methylene chloride/2 N methanolic ammonia) to afford 0.174 g (57%) of product as a white solid. Comparisons by ^{1}H NMR spectroscopy and analytical chiral HPLC (column: Chirex (S)-VAL and (R)-NE, 4.6×250 mm; eluant: 0.5% trifluoroacetic acid in 67:31:2 hexane/methylene chloride/ethanol; flow: 1 mL/min; detection 280 nM) demonstrate that this material was identical to a sample of the same compound prepared by the method of Polt, et al. (*J. Org. Chem.*, 1998, 63, 8837). Enantiomeric excess was determined to be 99.6%. Total contamination from the two possible diastereomers is determined to be 0.2%. ^{1}H NMR ($CDCl_3$) δ 6.88-6.73 (m, 3H), 5.84 (d, J=7.3, 1H), 4.90 (d, J=3.8, 1H), 4.24 (s, 4H), 4.22-4.15 (m, 1H), 2.86-2.72 (m, 2H), 2.72-2.55 (m, 4H), 2.10 (t, J=7.5, 2H), 1.82-1.74 (m, 4H), 1.58-1.46 (m, 2H), 1.32-1.16 (m, 24H), 0.88 (t, J=6.7, 3H) ppm.

Example 2

Large Scale Preparation of Ceramide-Like Compounds

(5S)-5-PHENYLMORPHOLIN-2-ONE

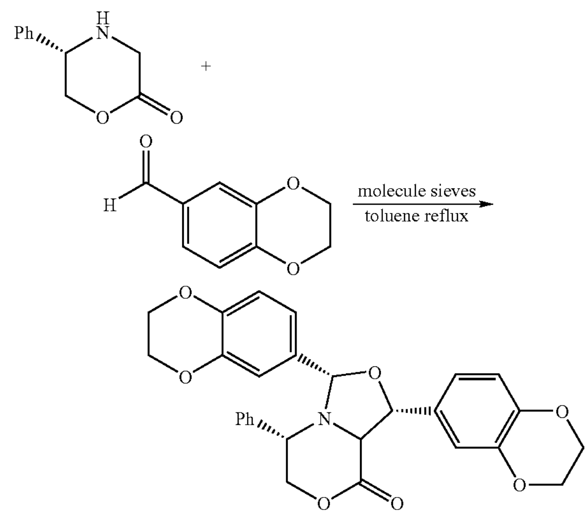


A solution of S-(+)-Phenyl glycinol (Aldrich, 10.17 g, 78.12 mmol) and Diisopropylethylamine (Aldrich, 34 mL, 195 mmol, 2.5 equivalents) was prepared in CH_3CN (200 mL). This solution was added to phenyl-α-bromoacetate (18.48 g, 85.9 mnol, 1.1 equivalents) dissolved in CH_3CN (50 mL) under nitrogen dropwise over 2 hours. The resulting solution was stirred under nitrogen for 16-20 hours. The solvent was removed by rotoevaporation keeping the bath

temperature at below 25° C. To the oil was added ethyl acetate (120 mL) and the mixture was stirred for 15 minutes. The resulting white precipitate was filtered off and the solid washed with ethyl acetate (25 mL). The filtrate was rotoevaporated to an oil keeping the bath temperature below 25° C. After drying under vacuum for 0.5 hours, the oil was dissolved in CH_2Cl_2 (17 mL) and loaded onto a silica gel column (60 g packed with 10% ethyl acetate/hexanes. The upper byproduct spots were eluted with 10% ethyl acetate/hexanes and the product was eluted with 50% ethyl acetate/hexanes-100% ethyl acetate. The fractions containing the product were rotoevaporated to an oil keeping the bath temperature below 25° C. This oil was dissolved in ethyl acetate (12 mL) and hexanes (60 mL) was added slowly in an ice bath to precipitate the product. The resulting precipitate was filtered. The white to yellow solid was vacuum dried. The (5S)-5-phenylmorpholin-2-one obtained (7.4 g, 41.8 mmol, 53%) was used directly in the next step.

Intermediate 1

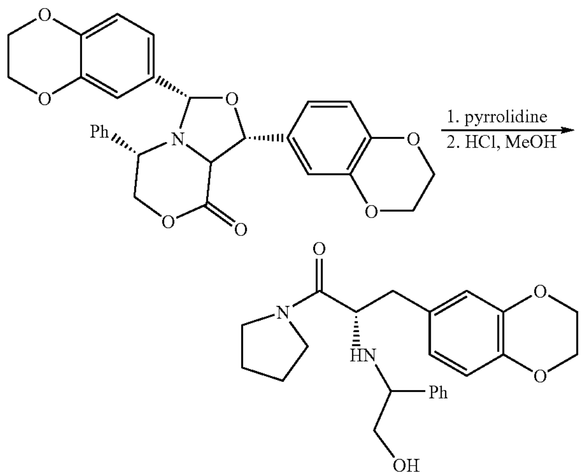
(1R,3S,5S,8aS)-1,3-Bis-(2',3'-dihydro-benzo[1,4] dioxin-6'-yl)-5-phenyl-tetrahydro-oxazolo[4,3-c][1,4]oxazin-8-one



(5S)-5-Phenylmorpholin-2-one (7.4 g, 41.8 mmol) and benzodioxolane-6-carboxaldehyde (Aldrich or Alfa Aesar, 20.56 g, 125.2 mmol, 3.0 equivalents) was dissolved in toluene (180 mL). The solution was placed in a soxhlet extractor apparatus filled with 4 □ molecular sieves (ca 30 g). The solution was refluxed under nitrogen for 2-3 days. After cooling to room temperature, the solvent was removed by rotoevaporation and the oil was dissolved in ethyl acetate (200 mL). A solution of sodium bisulfite (Aldrich, 50 g) in water (100 mL) was added and the two phase mixture was stirred at room temperature for 1 hour. The resulting white solid was filtered off and washed with ethyl acetate. The filtrate was placed in a separatory funnel and the layers separated. The organic layer was washed with water (100 mL) and saturated sodium chloride solution (100 mL). The dried (Na_2SO_4) solution was filtered and rotoevaporated to a yellow-red foamy oil (23.11 g). After drying under vacuum for 1 hour, diethyl ether (350 ml) was added and the mixture was stirred at room temperature for 16-20 hours. The resulting white-yellow solid was filtered. The solid was dried under vacuum. The cycloadduct was obtained in 46% yield (9.34 g).

Intermediate 2

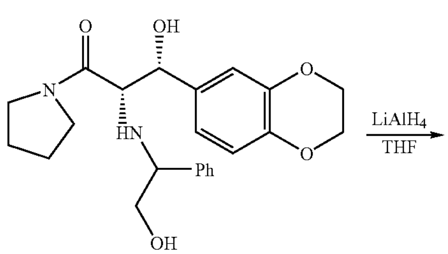
(2S,3R,1"S)-3-(2',3'-Dihydro-benzo[1,4]dioxin-6'-yl)-3-hydroxy-2-(2"-hydroxy-1"-phenyl-ethylamino)-1-pyrrolidin-1-yl-propan-1-one



To the cycloadduct (Intermediate 1, 6.7 g, 13.74 mmol) dissolved in methylene chloride (40 mL) was added pyrrolidine (Aldrich, 5.7 mL, 68.7 mmol, 5 equivalents). The solution was stirred under nitrogen at room temperature for 16-18 hours. The solvent was rotoevaporated to yield a yellow foamy oil which was vacuum dried for 0.5 hours. The crude was dissolved in methanol (115 mL) and a 1 M aqueous HCl solution (115 mL) was added. The solution was refluxed for 4 hours. After cooling to room temperature, the methanol was removed by rotoevaporation. Ethyl acetate (60 mL) was added and the two phase system was stirred at room temperature for 5-15 minutes. The two layers were separated and the organic layer was extracted with 1 M HCL (30 mL). The combined aqueous layers were washed two times with ethyl acetate (60, 30 mL). A saturated sodium bicarbonate solution (150 mL) was added to the aqueous layer slowly. The product was extracted three times with ethyl acetate (60 mL) from the basic (pH=8-9) aqueous layer. The combined organic layers containing the product were washed with a saturated sodium chloride solution (30 mL). After drying with Na_2SO_4 the solution was filtered and rotoevaporated to yield a yellow solid. Intermediate 2 was obtained in 93% yield (5.26 g).

Intermediate 3

(1R,2R,1"S)-1-(2',3'-Dihydro-benzo[1,4]dioxin-6'-yl)-2-(2"-hydroxy-1"-phenyl-ethylamino)-3-pyrrolidin-1-yl-propan-1-ol



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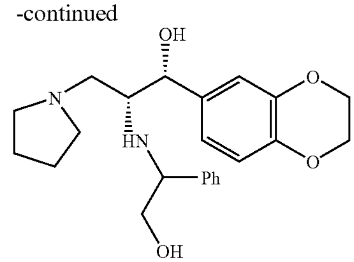
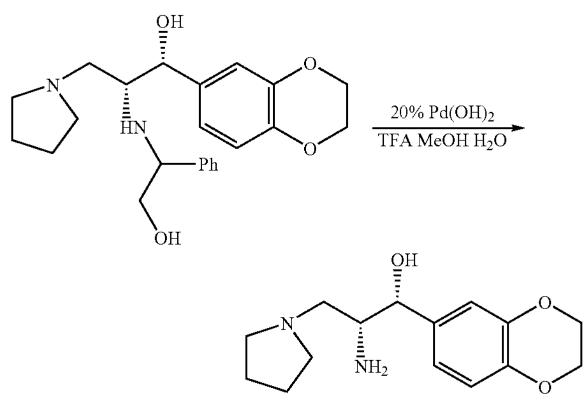


To a 3-neck flask equipped with a dropping funnel and condenser was added $LiAlH_4$ (Aldrich, 1.2 g, 31.7 mmol, 2.5 equivalents) and anhydrous THF (20 mL) under nitrogen. A solution of Intermediate 2 (5.23 g, 12.68 mmol) in anhydrous THF (75 mL) was added dropwise to the reaction over 15-30 minutes. The reaction was refluxed under nitrogen for 9 hours. The reaction was cooled in an ice bath and a 1M NaOH solution was carefully added dropwise. After stirring at room temperature for 15 minutes, water (50 mL) and ethyl acetate (75 mL) was added. The layers were separated and the aqueous layer was extracted twice with ethyl acetate (75 mL). The combined organic layers were washed with saturated sodium chloride solution (25 mL). After drying with Na_2SO_4 the solution was filtered and rotoevaporated to yield a colorless to yellow foamy oil. Intermediate 3 was obtained in 99% yield (5.3 g).

Intermediate 4

(1R,2R)-2-Amino-1-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-3-pyrrolidin-1-yl-propan-1-ol

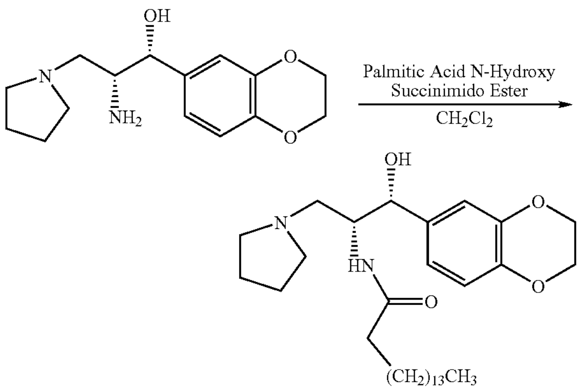


Intermediate 3 (5.3 g, 13.3 mmol) was dissolved in methanol (60 mL). Water (6 mL) and trifluoroacetic acid (2.05 mL, 26.6 mmol, 2 equivalents) were added. After being placed under nitrogen, 20% Palladium hydroxide on carbon (Pearlman's catalysis, Lancaster or Aldrich, 5.3 g) was added. The mixture was placed in a Parr Pressure Reactor Apparatus with glass insert. The apparatus was placed under nitrogen and then under hydrogen pressure 110-120 psi. The mixture was stirred for 2-3 days at room temperature under hydrogen pressure 100-120 psi. The reaction was placed under nitrogen and filtered through a pad of celite. The celite pad was washed with methanol (100 mL) and water (100 mL). The methanol was removed by rotoevaporation. The aqueous layer was washed with ethyl acetate three times (100, 50, 50 mL). A 10 M NaOH solution (10 mL) was added to the aqueous layer (pH=12-14). The product was extracted from the aqueous

layer three times with methylene chloride (100, 100, 50 mL). The combined organic layers were dried with Na_2SO_4, filtered and rotoevaporated to a colorless oil. The foamy oil was vacuum dried for 2 h. Intermediate 4 was obtained in 90 % yield (3.34 g).

Compound 5

(1R,2R)-Hexadecanoic acid [2-(2',3'-dihydro-benzo [1,4]dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-ylm-ethyl-ethyl]amide



To a solution of Intermediate 4 (3.34 g, 12.0 mmol) in methylene chloride (50 mL) was added a solution of palmitic acid N-hydroxylsuccinimide ester (Sigma, 4.24 g, 12.0 mmol) over 15-30 minutes under nitrogen at room temperature. The solution was stirred at room temperature for 18-20 hours. To the reaction was added methylene chloride (50 mL) and a 1 M NaOH solution (25 mL). The two phase system was stirred at room temperature for 15-30 min. Water (25 mL) was added and the layers were separated. The aqueous layer was back extracted with methylene chloride (25 mL). The combined organic layers were washed twice with water (25 mL) and once with a saturated sodium chloride solution (25 mL). The organic layer was dried with Na_2SO_4, filtered and rotoevaporated to a light yellow oil. The crude was recrystallized from hexane (50 mL). The white solid (5.46 g) obtained was separated on silica gel (300 g) with 2% methanol:methylene chloride—4% methanol:methylene chloride—4% 2 M ammonium in methanol:methylene chloride. The white solid obtained was recrystallized form hexanes (70 mL). Compound 5 was obtained in 66% yield (4.18 g). Analytical chiral HPLC (column: Chirex (S)-VAL and (R)-NE, 4.6×250 mm; eluant: 0.5% trifluoroacetic acid in 67:31:2 hexane/methylene chloride/ethanol; flow: 1 mL/min; detection: 280 nM) showed this material to be 98.98% pure with 0.89% of a diastereoisomer and 0.14% of the enantiomer.

Example 3

Alternative Large Scale Preparation of Ceramide-Like Compounds

(5S)-5- Phenylmorpholin-2-one HCl salt

A solution of phenyl bromoacetate (Aldrich, 862.17 g, 4.0 moles, 1.1 equivalents) in acetonitrile (reagent grade, 1500 ml) was cooled in an ice bath (internal temperature below 5° C.). To this was added a cold slurry (internal temperature below 5° C.) of S-(+)-2-phenyl glycinol (Aldrich, 500 g, 3.65 moles, 1 equivalent) and diisopropylethylamine (DIPEA) (Aldrich, 1587 ml, 9.11 moles, 2.5 equivalents) in acetonitrile (2900 ml) in portions while keeping the internal temperature below 10° C. The mixture was stirred at this temperature for 30 minutes before the ice bath was removed and the mixture was allowed to stir at room temperature for an additional 4 hours. The solvent was removed in vacuo while maintaining the bath temperature at 25° C. The mixture was coevaporated with ethyl acetate (2×500 ml) to produce a light yellow viscous oil. To the reaction mixture, ethyl acetate (4500 ml) was added and the flask was immersed in an ice bath with agitation. The mixture was allowed to cool below 8° C. The solid was filtered and washed with ethyl acetate (3×250 ml). The solution was cooled to below 5° C. Dry HCl gas was passed slowly into the solution while maintaining the internal temperature below 15° C. until the pH was below 2 (wet pH paper). The mixture was allowed to stir at this temperature and pH for an additional 20 minutes before the solid was suction filtered. The solid was washed with ethyl acetate (3×200 ml) and dried under high vacuum for about 20 hours. The yield was 412 g (53%). ^1H NMR was consistent with the (5S)-5- phenylmorpholin-2-one HCl salt.

Intermediate 1

(1R,3S,5S,8aS)-1,3-Bis-(2',3'-dihydro-benzo[1,4] dioxin-6'-yl)-5-phenyl-tetrahydro-oxazolo[4,3-c][1, 4]oxazin-8-one

To a stirred suspension of (5S)-5- phenylmorpholin-2-one HCl salt (381 g, 1 equivalent) in 15% ethyl acetate in toluene (2270 ml) was added a solution of sodium bicarbonate (1.1 equivalents) in water (2000 ml). The resulting biphasic solution was stirred at room temperature for about 1 hour. The organic layer was transferred to a flask containing 1,4-benzodioxan-6-carboxaldehyde. The flask was then equipped with a Dean-Stark unit, a condenser and a nitrogen inlet. The mixture was heated at reflux with agitation while about 650 ml solvent (mixture of ethyl acetate and toluene) was collected via Dean-Stark unit. The resulting yellow-red solution was allowed reflux for about 64 hours, under nitrogen while the water formed during the reaction was collected in the Dean-Stark unit. Most of the solvent was then removed via distillation at atmospheric pressure through Dean-Stark unit. The residual solvent was then removed by coevaporation with heptane (500 ml) and tert-butylmethyl ether (2×725 ml) to produce a yellow semi solid product. The semi solid product was dissolved in ethyl acetate (3400 ml). A solution of sodium bisulfite (920 g) in water (1500 ml) was added and the mixture was allowed to stir at room temperature for about 1 hour. The solid that was formed was removed by filtration and washed with ethyl acetate (3×400 ml). The filtrate was washed with water (1450 ml), 5% brine solution (1450 ml) and dried over $MgSO_4$ (100 g). The solvent was removed in vacuo to afford a yellow solid. To this was added tert-butylmethyl ether (2900 ml) and the suspension was stirred at room temperature for 20 to 22 hours. The yellow solid was suction filtered, washed with tert-butylmethyl ether (2×600 ml) and dried under high vacuum at room temperature for about 22 hours. The yield was 400.5 g (58%). ^1H NMR and TLC were consistent with Intermediate 1.

Intermediate 2

2S,3R,1"S)-3-(2',3'-Dihydro-benzo[1,4]dioxin-6'-yl)-3-hydroxy-2-(2"-hydroxy-1"-phenyl-ethylamino)-1-pyrrolidin-1-yl-propan-1-one

A solution of Intermediate 1 (312 g, 0.64 moles), pyrrolidine (267 ml, 3.2 moles, 5 equivalents) and tetrahydrofuran (1350 ml) was heated at reflux for 4.5 hours under nitrogen atmosphere. The solvent and excess pyrrolidine were removed in vacuo to produce the crude intermediate as an orange viscous oil. The oil was dissolved in methanol (3000 ml) and 1M hydrochloric acid solution (3000 ml). The resulting solution was heated at reflux for about 7 hours. The solvent was then removed in vacuo to afford a mixture of an oil and water. To this ethyl acetate (2000 ml) was added and the aqueous layer was separated. The organic layer was extracted with aqueous 1M HCl (1000 ml). The aqueous layers were combined and washed with ethyl acetate (2000 ml). The aqueous layer was cooled in an ice bath. The pH of the aqueous layer was adjusted to about 9 (pH paper) with 10 M aqueous NaOH (525 ml). The aqueous layer was extracted with ethyl acetate (3000 ml). The organic layer was washed with 5% brine solution (1000 ml) and dried (Na_2SO_4). The solvent was removed in vacuo to produce a yellow viscous oil. The yield was 213.4 g, 81%. ^{1}H NMR was consistent with Intermediate 2.

Intermediate 3

1R,2R,1"S)-1-(2',3'-Dihydro-benzo[1,4]dioxin-6'-yl)-2-(2"-hydroxy-1"-phenyl-ethylamino)-3-pyrrolidin-1-yl-propan-1-ol

To a slurry of $LiAlH_4$ (50.7 g, 1.34 moles, 2.6 equivalents) in tetrahydrofuran (700 ml) was added a solution of Intermediate 2 (213.34 g, 0.517 moles) in tetrahydrofuran (2000 ml) slowly with agitation at room temperature. The mixture was refluxed for about 4 hours. TLC analysis (10% methanol in methylene chloride, v/v) indicated consumption of the starting material. The reaction mixture was cooled in an ice bath (below 5° C.) and water (135 ml) was added very slowly while keeping the internal temperature less than or equal to 10° C. To this was then added a 15% aqueous NaOH solution (70 ml) followed by water (200 ml). The reaction mixture was allowed to warm to room temperature while the agitation was continued. Methylene chloride (1000 ml) was then added to the mixture and the salts were filtered through a pad of celite. The salts were washed with methylene chloride (2×500 ml). The filtrates were combined and the solvent was removed in vacuo to produce a yellow oil. The oil was dissolved in 1M aqueous HCl (1500 ml) and washed with ethyl acetate (3×500 ml). The aqueous layer was cooled in an ice bath to below 5° C. and the pH of the aqueous layer was adjusted to 12 to 13 with a 10 M aqueous NaOH solution (220 ml) keeping the internal temperature at less than or equal to 10° C. The mixture was allowed to warm to room temperature. The aqueous layer was extracted with methylene chloride (2×500 ml). The organic layers were combined and washed with brine solution (500 ml), dried (Na_2SO_4) and the solvent was removed in vacuo to afford a yellow viscous oil. The yield was 186.4 g (88.5%). ^{1}H NMR was consistent with Intermediate 3.

Intermediate 4 dioxalate salt

(1R,2R)-2-Amino-1-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-3-pyrrolidin-1-yl-propan-1-ol dioxalate salt

A suspension of Intermediate 3 (358 g, 0.90 moles), ethanol (1500 ml), 1M HCl solution (1500 ml) and 10% $Pd(OH)_2$ (32 g, 20 weight %) were hydrogenated at about 50 psi for about 36 h at room temperature. The mixture was filtered through a Cuono filter. The Cuono filter was washed with 10% ethanol in water (500 ml). The filtrates were combined and ethanol was removed in vacuo. The aqueous layer was extracted with ethyl acetate (3×600 ml). The organic layer was extracted with 1M HCl aqueous (700 ml). The aqueous layers were combined and cooled in an ice bath (0 about 5° C.). The pH of the aqueous layer was adjusted to about 12 (pH paper) with 10M aqueous NaOH solution (490 ml) keeping the internal temperature below 10° C. The aqueous layer was allowed to warm to room temperature. The aqueous layer was extracted with methylene chloride (2×1500 ml, 1×750 ml). The combined organic layers were dried over $MgSO_4$ and the solvent was removed in vacuo to afford a yellow viscous oil. The crude weight was 214.3 g (86%). ^{1}H NMR was consistent with Intermediate 4.

A solution of oxalic acid (152.4 g, 1.693 moles, 2.2 equivalents) in methylisobutyl ketone (2300 ml) was added slowly with stirring to a solution of Intermediate 4 (214.3 g, 0.77 moles, 1 equivalent) in methylisobutyl ketone (800 ml) at room temperature. The resulting mixture was stirred at room temperature for about 2.5 hours. The solid was filtered, and triturated with acetone (2000 ml) at room temperature for about 16 hours. The solid was filtered, washed with acetone (3×100 ml) and dried under high vacuum to produce an off-white solid. The yield was 312.5 g (89%). ^{1}H NMR was consistent with Intermediate 4 dioxalate salt.

Compound 5

(1R,2R)-Hexadecanoic acid [2-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-ylm-ethyl-ethyl]-amide

To a cold solution (about 5° C.) of Intermediate 4 dioxalate salt (507 g, 1.11 moles) in water (10 L) was added a 10 M aqueous NaOH solution (500 ml) with stirring while keeping the internal temperature below 10° C. The solution was allowed to warm to room temperature while the pH of the solution was maintained at about 14 (pH paper). The aqueous layer was extracted with methylene chloride (3×6000 ml). The organic layers were combined, washed with water (2000 ml), dried ($MgSO_4$) and the solvent was removed in vacuo to afford a yellow viscous oil, Intermediate 4. The yield was 302 g (98%). ^{1}H NMR was consistent with Intermediate 4.

A solution of palmitic acid NHS-ester (Sigma, 382.5 g, 1.01 equivalents) in methylene chloride (2500 ml) was added to a solution of intermediate 4 (302 g) in methylene chloride (1500 ml) at room temperature over a period of 1.25 hours under a nitrogen atmosphere. The mixture was allowed to stir at room temperature for about 18 hours. A solution of 1M aqueous NaOH (2425 ml) was added and the mixture was stirred at room temperature for about 3 hours. The organic layer was separated and the aqueous layer was extracted with methylene chloride (800 ml). The organic layers were combined, washed with a 1M NaOH solution (3×1500 ml) and water (1500 ml). The organic layer was dried over $MgSO_4$ and the solvent was removed in vacuo to afford a semi solid. The semi-solid was coevaporated with heptane (3×100 ml). The crude product was transferred to a 12 L three-necked RB flask and heptane (7500 ml) was added. The mixture was heated at reflux with stirring under a nitrogen atmosphere. The solution was slowly cooled to about 55° C. (internal temperature) and poured into another flask. The solution was stirred at room temperature for 24 hours under a nitrogen atmosphere. The off white solid was filtered, washed with heptane (2×500 ml) and dried under high vacuum for 24

hours. The solid (397 g) was transferred to a 12 L RB flask and 30% ethyl acetate in heptane (8000 ml) was added. The resulting mixture was heated at reflux for 30 minutes with stirring. The solution was cooled to about 55° C. (internal temperature) and poured into another flask. The stirring was continued at room temperature under a nitrogen atmosphere for about 24 hours. The solid was filtered, washed with heptane (2×100 ml) and dried under high vacuum to afford an off white solid. The yield was 324 g (58%). ^{1}H NMR and TLC were consistent with Compound 5. mp 96.1° C. HPLC analysis: chiral purity 99.7%, chemical purity 99.7%. Anal. Calcd for $C_{31}H_{52}N_2O_4$:C, 72.05; H, 10.14; N, 5.42. Found C, 72.03; H, 10.19; N, 5.42.

Example 4

Preparation of Compounds 6-8

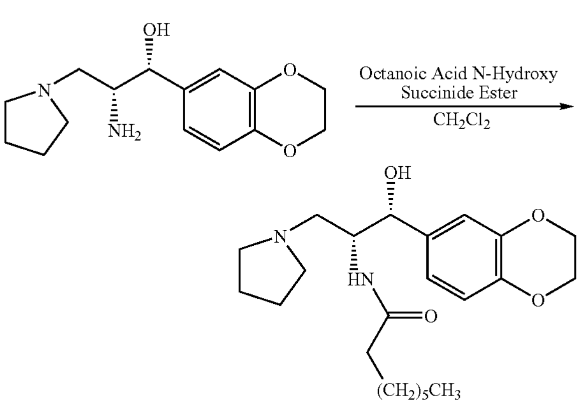
N-hydroxysuccinimide esters of fatty acids were prepared by the method of Lapidot, Y. Rappoport, S. and Wolman, Y. *Journal of Lipid Research* 8, 1967 or as described below:

Octanoic Acid N-Hydroxysuccinimide Ester

N-hydroxysuccinimide (Aldrich, 20.0 g, 173 mmol) and triethyl amine (29 mL, 208 mmol) were dissolved in methylene chloride in an ice bath under nitrogen. Octanoyl chloride (Aldrich, 35 mL, 205 mmol) was added dropwise over 0.5 hours. The ice bath was removed and the solution with a white solid was stirred for 1 hour at room temperature. The white solid was removed by filtration and the filtrate was washed with water (100 mL) and saturated aqueous sodium bicarbonate (100 mL). The organic layer was dried with sodium sulfate, filtered and heptane (100 mL) was added. The solution was rotoevaporated to remove most of the methylene chloride and leave a colorless to white flaky precipitate in heptane. The precipitate was filtered and washed with heptane. After drying, Octanoic acid N-hydroxysuccinimide ester was obtained in 84% yield (35.4 g).: ^{1}H NMR ($CDCl_3$) 2.84 (br s, 4H), 2.60 (t, J=7.48 Hz, 2H), 1.78-1.71 (m, 2H), 1.42-1.26 (m, 8H), 0.88 (t, J=6.7 Hz, 3H) ppm.

Compound 6

(1R,2R)-Octanoic acid [2-(2',3'-dihydro-benzo[1,4] dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide

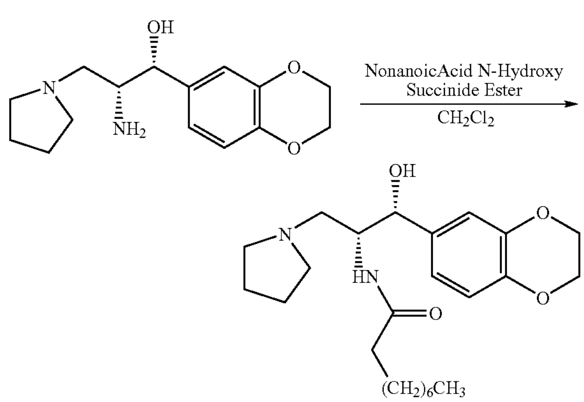


Compound 6

To Intermediate 5 (22.36 g, 80.33 mmol) dissolved in anhydrous methylene chloride (300 mL) was added a solution of octanoic acid N-hydroxysuccinimide ester (19.4 g, 80.39 mmol) dissolved in anhydrous methylene chloride (150 mL) over 15-30 minutes under nitrogen at room temperature. The solution was stirred at room temperature for 18-20 hours. To the reaction was added 1M aqueous NaOH solution (200 mL). The two phase system was stirred at room temperature for 45 minutes. The layers were separated and the combined organic layers were washed twice with 1M NaOH (2×200 mL) and twice with water (2×100 mL). The organic layer was dried with sodium sulfate, filtered and rotoevaporated to a yellow oil. Most of the crude material was dissolved in 5% ethyl acetate in heptane (1 L) at reflux. After cooling to 40° C., the hazy solution was separated from the yellow oil by decanting the solution into a new flask. The first flask was rinsed twice with 5% ethyl acetate in heptane (2×250 mL) by the same process (reflux and cooling to 40° C. and decanting the solution from the oil). The combined solution was heated to reflux and allowed to cool to room temperature over 4 hours. The resulting white solid was filtered and washed with 5% ethyl acetate in heptane (100 mL) and heptane (100 mL). The white solid (13.9 g) was dried under vacuum for 16-24 hours. This solid was mostly dissolved in 5% ethyl acetate in heptane (800 mL) at reflux. After cooling to 50° C., the hazy solution was separated from the yellow oil by decanting the solution into a new flask. The first flask was rinsed with 5% ethyl acetate in heptane (100 mL) by the same process (reflux and cooling to 50° C. and decanting the solution from the oil). The combined solution was heated to reflux and allowed to cool to room temperature over 4 hours. The resulting white solid was filtered and washed with 5% ethyl acetate/heptane (50 mL) and heptane (50 mL). After drying at room temperature under vacuum for 2-3 days, Compound 6 was obtained in 39% yield (12.57 g). Analytical chiral HPLC (column: Chirex (S)-VAL and (R)-NE, 4.6×250 mm) showed this material to be 99.9% the desired R,R isomer. Analytical HPLC showed this material to be 99.6% pure. mp 87-88° C. ^{1}H NMR ($CDCl_3$) δ 6.86-6.73 (m, 3H), 5.84 (d, J=7.3 Hz, 1H), 4.91 (d, J=3.4 Hz, 1H), 4.25 (s, 4H), 4.24-4.18 (m, 1H), 2.85-2.75 (m, 2H), 2.69-2.62 (m, 4H), 2.10 (t, J=7.3 Hz, 2H), 1.55-1.45 (m, 2H), 1.70-1.85 (m, 4H), 1.30-1.15 (m, 8H), 0.87 (t, J=6.9 Hz, 3H) ppm.

Compound 7

(1R,2R)-Nonanoic acid [2-(2',3'-dihydro-benzo[1,4] dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide



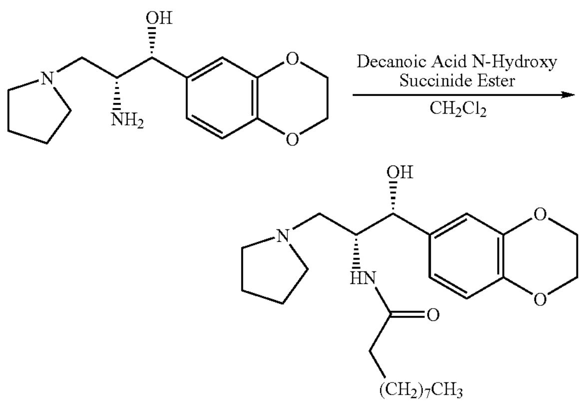
Compound 7

This compound was prepared by the method described for Compound 6 using Nonanoic acid N-hydroxysuccinimide

ester. Analytical HPLC showed this material to be 98.4 % pure. mp 74-75° C. ^{1}H NMR ($CDCl_3$) δ 6.86-6.76 (m, 3H), 5.83 (d, J=7.3 Hz, 1H), 4.90 (d, J=3.3 Hz, 1H), 4.24 (s, 4H), 4.24-4.18 (m, 1H), 2.85-2.75 (m, 2H), 2.69-2.62 (m, 4H), 2.10 (t, J=7.3 Hz, 2H), 1.55-1.45 (m, 2H), 1.70-1.85 (m, 4H), 1.30-1.15 (m, 10H), 0.87 (t, J=6.9 Hz, 3H) ppm.

Compound 8

(1R,2R)-Decanoic [2-(2',3'-dihydro-benzo[1,4]dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide



Compound 8

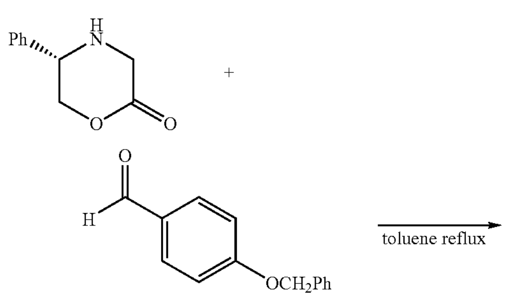
This compound was prepared by the method described for Compound 6 using decanoic acid N-hydroxysuccinimide ester. Analytical HPLC showed this material to be 99.3 % pure. mp 97.5-98.5° C. ^{1}H NMR ($CDCl_3$) δ 6.86-6.76 (m, 3H), 5.83 (d, J=7.5 Hz, 1H), 4.90 (d, J=3.4 Hz, 1H), 4.24 (s, 4H), 4.24-4.18 (m, 1H), 2.85-2.75 (m, 2H), 2.69-2.62 (m, 4H), 2.10 (t, J=7.5 Hz, 2H), 1.55-1.45 (m, 2H), 1.70-1.85 (m, 4H), 1.30-1.15 (m, 12H), 0.87 (t, J=6.8 Hz, 3H) ppm.

Example 5

Preparation of Compound 13

Intermediate 9

(1R,3S,5S,8aS)-1,3-Bis-(4-benzyloxy-phenyl)-5-phenyl-tetrahydro-oxazolo[4,3-c][1,4]oxazin-8-one



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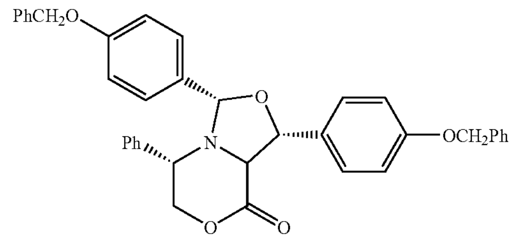
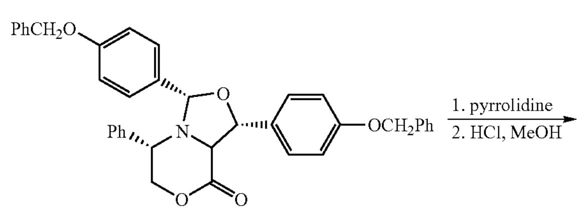


The (5S)-5-phenylmorpholin-2-one HCl salt (57.45, 268.9 mmol) was stirred with ethyl acetate (500 mL) and saturated aqueous sodium bicarbonate (250 mL) for 30 minutes, until the biphasic solution was clear. The phases were separated, and the aqueous layer was extracted with ethyl acetate (2×250 mL). The combined organic phases were washed with saturated sodium chloride solution (250 mL). The organic layer was dried with sodium sulfate, filtered, concentrated to an oil, and dried under vacuum for 60 minutes. The 5-(S)-phenyl morpholin-2-one was obtained in a 86% yield (40.98 g, 231.3 mmol).

The 5-(S)-phenyl morpholin-2-one (40.98 g, 231.3 mmol) and 4-benzyloxybenzaldehyde (Aldrich, 147.3 g, 694 mmol, 3.0 equivalents) was dissolved in toluene (750 mL). The reaction was fitted with a Dean Stark Trap and a reflux condenser. The solution was refluxed under nitrogen for 2 days. After cooling to room temperature, the solvent was removed by rotoevaporation and the oil was dissolved in ethyl acetate (500 mL). A solution of sodium bisulfite (Aldrich, 125 g) dissolved in water (250 mL) was added and the two phase mixture was stirred at room temperature for 3 hours. The resulting white solid was filtered off and washed with ethyl acetate. The filtrate was placed in a separatory funnel and the layers separated. The organic layer was washed with water (250 mL), saturated aqueous sodium chloride solution (250 mL) and then dried (sodium sulfate) filtered and rotoevaporated to a foamy oil (144 g). After drying under vacuum for 1 hour, tert-butyl methyl ether (1450 mL) was added and the mixture was stirred at room temperature for 5 hours. The resulting white-yellow solid was filtered. The solid was dried under vacuum. Intermediate 9 was obtained in 27% yield (41.64 g, 71.46 mmol). ^{1}H NMR ($CDCl_3$) δ 7.5-6.8 (m, 23H), 5.0 and 5.1 (2 s, 4H), 4.5-4.3 (m, 2H), 4.2-4.1 (m, 2H) ppm.

Intermediate 10

(2S,3R,1"S)-3-(4-Benzyloxy-phenyl)-3-hydroxy-2-(2"-hydroxy-1"-phenyl-ethylamino-1-pyrrolidin-1-yl-propan-1-one



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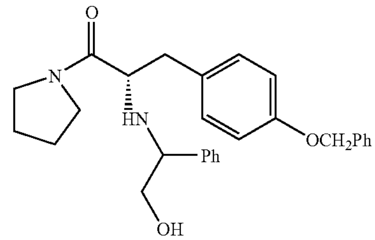
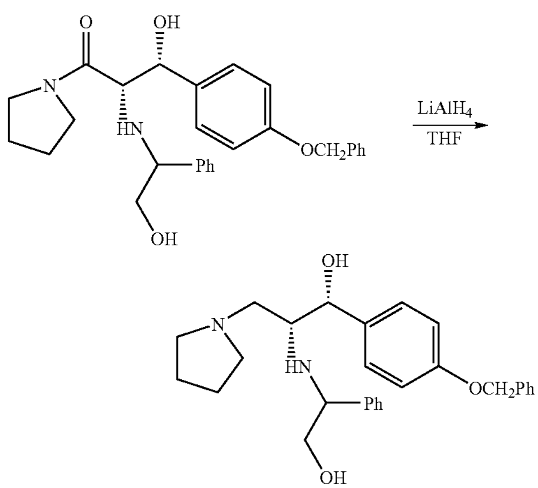


To Intermediate 9 (45.1 g, 77.4 mmol) dissolved in tetrahydrofuran (250 mL) was added pyrrolidine (Aldrich 33 mL, 395 mmol, 5.1 equivalents). The solution was stirred capped under nitrogen at room temperature for 16-18 hours. The solvent was rotoevaporated to yield a yellow foamy oil which was vacuum dried for 0.5 hours. The crude was dissolved in methanol (220 mL) and a 1 M aqueous HCl solution (220 mL) was added. The solution was refluxed for 4 hours. After cooling to room temperature, the methanol was removed by rotoevaporation. To the resulting oil was slowly added 10 M aqueous NaOH (22 mL to adjust the pH to 14). The produce was extracted three times with methylene chloride (300, 100, 100 mL) from the basic aqueous layer. After drying with sodium sulfate the combined organic layer was filtered and rotoevaporated to yield a yellow-orange foamy solid. Tert-butyl methyl ether (300 mL) was added and the mixture was stirred at room temperature for 7 hours. The resulting white-yellow solid was filtered, washed with tert-butyl methyl ether (50 mL) and vacuum dried. Intermediate 10 was obtained in 83% yield (29.77 g). ^{1}H NMR ($CDCl_3$) δ 7.4-7.2 (m, 12H), 6.9-6.8 (m, 2H), 5.05 (AB quartet, 2H), 4.47 (d, J=8.5, 1H), 3.9-3.3 (m, 3H), 3.05 (d, J=8.5, 1H), 3.0-2.8 (m, 2H), 2.3-2.2 (m, 1H), 1.85-1.7 (m, 1H), 1.45-1.15 (m, 4H) ppm.

Intermediate 11

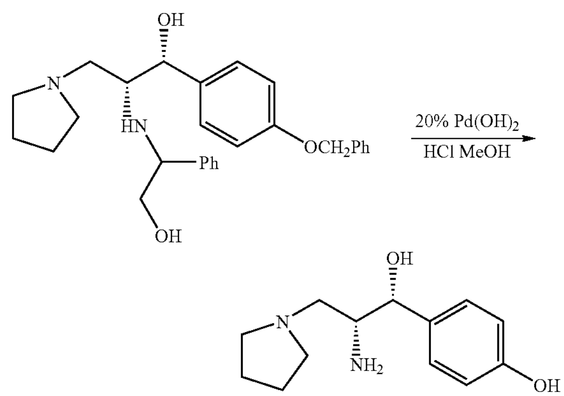
(1R,2R,1"S)-1-(4-Benzyloxy-phenyl)-2-(2"-hydroxy-1"-phenyl-ethylamino)-3-pyrrolidin-1-yl-propan-1-ol



In a 3-neck flask with dropping funnel and condenser under nitrogen was added $LiAlH_4$ (Aldrich, 6.3 g, 166 mmol, 2.57 equivalents) and anhydrous tetrahydrofuran (75 mL). A solution of Intermediate 10 (2.9.7 g, 64.48 mmol) in anhydrous tetrahydrofuran (300 mL) was added dropwise to the reaction over 15-30 minutes. The reaction was refluxed under nitrogen for 9 hours. The reaction was cooled in an ice bath and water (7.0 mL) was very carefully added drop by drop (vigorous exothermic reaction with hydrogen being given off). A 15% aqueous NaOH solution (7.0 mL) was added dropwise followed by water (21 mL). Halfway through the final water addition a large amount of a white solid formed. It was broken up by the addition of methylene chloride (250 mL). After stirring at room temperature for 15 minutes, the mixture was filtered through a celite plut (17 cm in diameter by 1 cm in height). The precipitate was washed with methylene chloride (2×250 mL). The filtrate was rotoevaporated to an oil. The oil was dissolved in 1M aqueous HCl (300 mL). This aqueous layer was washed with tert-butyl methyl ether (2×200 mL). After cooling in an ice bath, 10 M aqueous NaOH (35 mL) was carefully added to the aqueous layer (final pH=14). The product was extracted three times with methylene chloride (300 mL, 200 mL and 100 mL). After drying with sodium sulfate, the solution was filtered and rotoevaporated to yield a white solid. After drying, the Intermediate 11 was obtained in 94% yield (26.9 g).). ^{1}H NMR ($CDCl_3$) δ 7.46-7.115 (m, 12H), 6.98-6.96 (m, 2H), 5.08 (s, 2H), 4.49 (d, J=4.7, 1H), 3.70-3.65 (m, 1H), 3.60-3.55 (m, 1H), 3.54-3.45 (m, 1H), 3.00-2.90 (m, 1H), 2.7-2.6 (m, 1H), 2.36 (br s, 4H), 2.15-2.05 (m, 1H), 1.70 (br s, 4H) ppm.

Intermediate 12

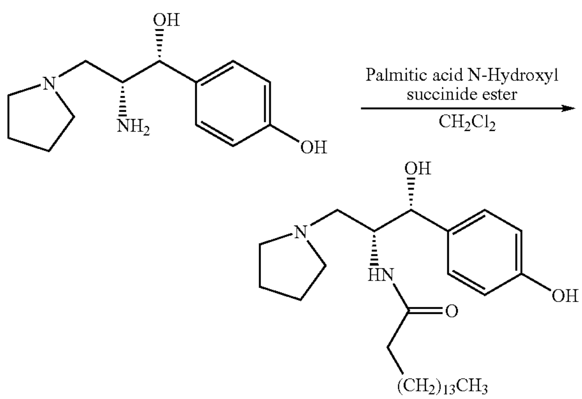
(1R,2R)-2-Amino-1-(4-benzyloxy-phenyl)-3-pyrrolidin-1-yl-propan-1-ol Hydrogen chloride salt



Intermediate 11 (26.9 g, 60.24 mmol) was dissolved in methanol (400 mL) and 1M aqueous HCl (130 mL) was added. After being placed under nitrogen, 20% palladium hydroxide on carbon (Pearlman's catalysis, Aldrich, 10.8 g) was added. The reaction was placed under nitrogen and then under hydrogen by evacuation and filling to a balloon. The mixture was stirred for 48 hours at room temperature under a hydrogen balloon. The reaction was placed under nitrogen and filtered through a pad of celite. The celite pad was washed with 10% water in methanol (250 mL) and water (mL). The solvent was removed by rotoevaporation and coevaporation with toluene (3×100 mL). The foamy solid was dissolved in isopropanol (300 mL) at reflux. The solution was cooled to room temperature and tert-butyl methyl ether (550 mL) was added. After stirring at room temperature for 2 hours, the white solid was filtered and washed with tert-butyl methyl ether. After drying, Intermediate 12 was obtained in ca 99% yield (18 g).). ^{1}H NMR (DMSO-d6) δ 9.68 (br s, 1H), 8.53 (br s, 2H) 7.24 (d, J=8.55 Hz, 2H), 6.80 (d, J=8.55 Hz, 2H), 4.72 (d, J=7.0 Hz, 1H), 3.8-3.6 (m, 2H), 3.4-3.6 (m, 3H), 3.0-3.2 (m, 2H), 2.7-2.5 (br s, 1H), 2.0-1.7 (br s, 4H) ppm.

Compound 13

(1R,2R)-Hexadecanoic acid [2-(4-benzyloxy-phenyl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide

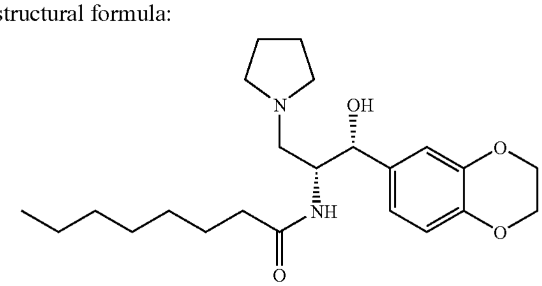


To Intermediate 12 (16.17 g 49.36 mmol) suspended in tetrahydrofuran (500 mL) was added triethylamine (28 mL, 4 equivalents). A solution of Palmitic acid N-hydroxysuccinimide ester (Sigma, 19.2 g, 54.29 mmol) dissolved in tetrahydrofuran (125 mL) was added over 30 minutes under nitrogen at room temperature. The solution was stirred at room temperature for 18-20 hours. The white precipitate was removed by filtration and the filtrate was rotoevaporated to a foamy off-white solid (35.5 g). The crude material was dissolved in methylene chloride (500 mL) and washed with water (100 mL) and saturated aqueous sodium carbonate solution (100 mL). After drying with sodium sulfate, the solution was filtered and rotoevaporated to yield a off-white foamy solid (24.75 g). This material was recrystallized from 40% ethyl acetate in heptane (500 mL, hot filtration). Compound 13 was obtained in 61% yield (14.45 g) Analytical chiral HPLC showed this material to be 99.7% the desired R,R isomer. Analytical HPLC showed this material to be 99.6% pure. mp 95-97° C. ^{1}H NMR ($CDCl_3$) δ 7.15 (d, J=8.5 Hz, 2H), 6.70 (d, J=8.5 Hz, 2H), 6.0 (d, J=7.3, 1H), 4.96 (d, J=3.8, 1H), 4.3-4.2 (m, 1H), 2.9-2.7 (m, 2H), 2.65-2.55 (m, 4H), 2.10 (t, J=7.5, 2H), 1.75 (br s, 4H), 1.58-1.46 (m, 2H), 1.32-1.16 (m, 24H), 0.9 (t, J=6.7, 3H) ppm.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

What is claimed is:

1. A method of inhibiting glucosylceramide synthase or lowering glycosphingolipid concentrations in a subject in need thereof, comprising administering to the subject an effective amount of a compound represented by the following structural formula:



or a physiologically acceptable salt thereof.

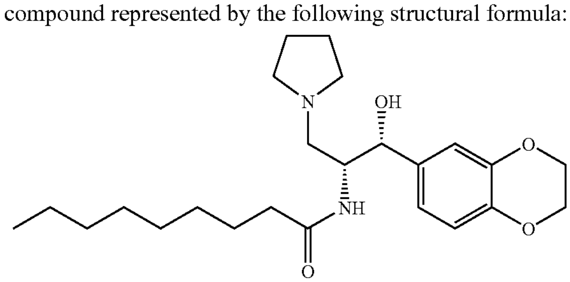
2. The method of claim **1**, wherein the compound has an enantiomeric excess of at least 25%.

3. The method of claim **1**, wherein the compound has an enantiomeric excess of at least 50%.

4. The method of claim **1**, wherein the compound has an enantiomeric excess of at least 90%.

5. The method of claim **1**, wherein the compound has an enantiomeric excess of at least 99%.

6. A method of treating a subject with Fabry disease, comprising administering to the subject an effective amount of a compound represented by the following structural formula:



or a physiologically acceptable salt thereof.

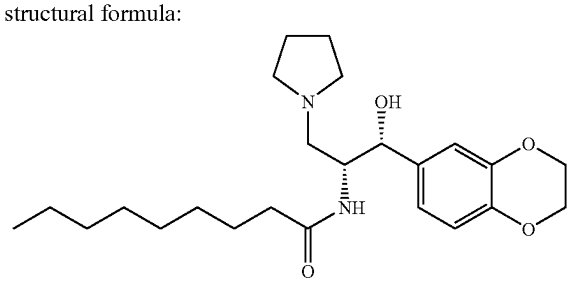
7. The method of claim **6**, wherein the compound has an enantiomeric excess of at least 25%.

8. The method of claim **6**, wherein the compound has an enantiomeric excess of at least 50%.

9. The method of claim **6**, wherein the compound has an enantiomeric excess of at least 90%.

10. The method of claim **6**, wherein the compound has an enantiomeric excess of at least 99%.

11. A method of inhibiting glucosylceramide synthase or lowering glycosphingolipid concentrations in a subject in need thereof, comprising administering to the subject an effective amount of a compound represented by the following structural formula:



or a physiologically acceptable salt thereof.

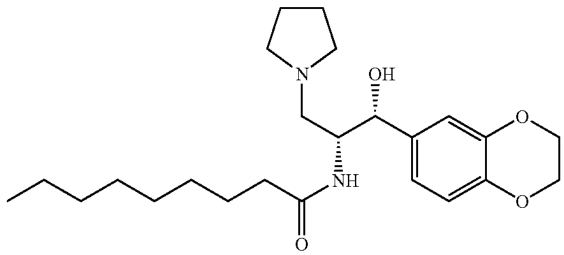
12. The method of claim **11**, wherein the compound has an enantiomeric excess of at least 25%.

13. The method of claim **11**, wherein the compound has an enantiomeric excess of at least 50%.

14. The method of claim **11**, wherein the compound has an enantiomeric excess of at least 90%.

15. The method of claim **11**, wherein the compound has an enantiomeric excess of at least 99%.

16. A method of treating a subject with Fabry disease, comprising administering to the subject an effective amount of a compound represented by the following structural formula:



or a physiologically acceptable salt thereof.

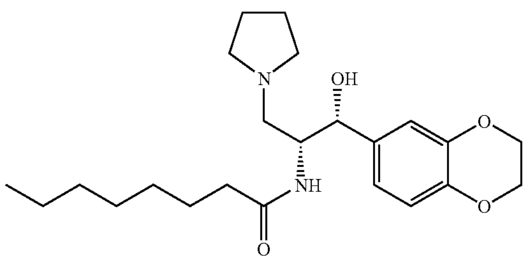
17. The method of claim **16**, wherein the compound has an enantiomeric excess of at least 25%.

18. The method of claim **16**, wherein the compound has an enantiomeric excess of at least 50%.

19. The method of claim **16**, wherein the compound has an enantiomeric excess of at least 90%.

20. The method of claim **16**, wherein the compound has an enantiomeric excess of at least 99%.

21. A method of treating a subject with Gaucher disease, comprising administering to the subject an effective amount of a compound represented by the following structural formula:



or a physiologically acceptable salt thereof.

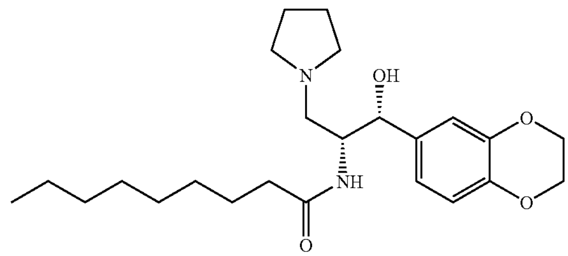
22. The method of claim **21**, wherein the compound has an enantiomeric excess of at least 25%.

23. The method of claim **21**, wherein the compound has an enantiomeric excess of at least 50%.

24. The method of claim **21**, wherein the compound has an enantiomeric excess of at least 90%.

25. The method of claim **21**, wherein the compound has an enantiomeric excess of at least 99%.

26. A method of treating a subject with Gaucher disease, comprising administering to the subject an effective amount of a compound represented by the following structural formula:



or a physiologically acceptable salt thereof.

27. The method of claim **26**, wherein the compound has an enantiomeric excess of at least 25%.

28. The method of claim **26**, wherein the compound has an enantiomeric excess of at least 50%.

29. The method of claim **26**, wherein the compound has an enantiomeric excess of at least 90%.

30. The method of claim **26**, wherein the compound has an enantiomeric excess of at least 99%.

* * * * *

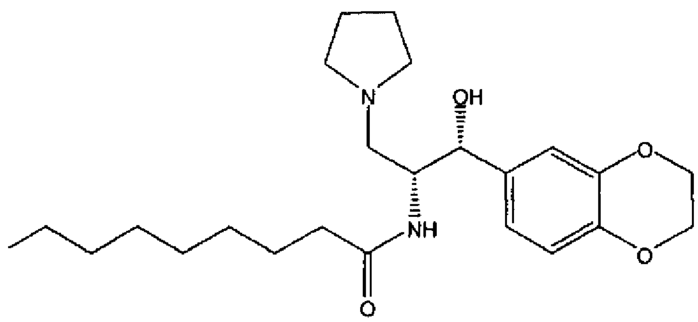
UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

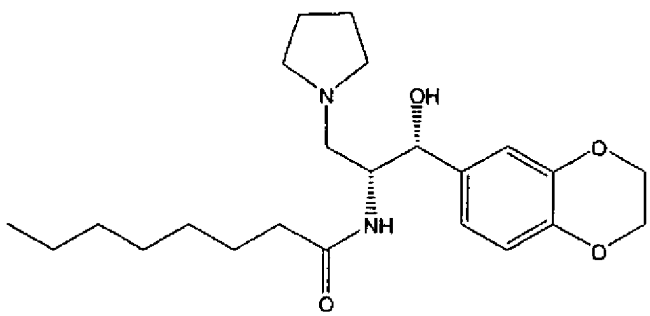
PATENT NO. : 7,615,573 B2
APPLICATION NO. : 11/702425
DATED : November 10, 2009
INVENTOR(S) : Craig Siegel et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 30, Claim 6, lines 15-21, delete

“  ”

and insert

--  --

Signed and Sealed this

Second Day of February, 2010

David J. Kappos

David J. Kappos
Director of the United States Patent and Trademark Office